Journal of
DAIRY SCIENCE

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Manuscripts for publication in the Journal of Dairy Science should be submitted to the editor, E. O. Herreid, at the address above. See January, 1954, issue, page 103, for information concerning the preparation of manuscripts, or send for reprint, "Information for Authors."

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PEOPLE AND EVENTS

UNIVERSITY NEWS

Michigan

C. F. Huffman Retires

DR. C. F. HUFFMAN, one of the most distinguished dairy scientists in the world, retired at Michigan State University on July 1.

C. F. Huffman

Dr. Huffman's service of 39 yr. was marked by outstanding contributions in nutrition research. Few university professors have received more honors and awards. In 1937, he received the first Borden Award in Dairy Production. Seventeen years later, in 1952, it was the American Feed Manufacturers' Award. In 1961, he was the recipient of the Award of Honor from the A.D.S.A.

Dr. Huffman's valuable research and contributions span a long period, but they are not to be entirely terminated with retirement. In August this year he will appear on the program of the Tenth Pacific Science Congress Conference at Honolulu.

Last year Michigan State University selected Doctor Huffman for a Distinguished Faculty Award, presenting him with a check for $1,000.

Dairy Majors Receive Awards

At the annual honors convocation of the College of Agriculture for 1961, two dairy seniors received outstanding recognition. CAMILLA CRISTMAN received the All-College Activities Award. She is the first woman ever to be so designated. Miss Cristman was cited for her activities in the Dairy Club, having served it as secretary and as club representative on the Agricultural Club. She was vice-president of the Judiciary Board of A.W.S. (Associated Women Students), Captain of Circle Honorary, and served on the Council of the dormitory. At the meeting of the Student Branch of A.D.S.A. at Madison in June, Miss Cristman was elected secretary for the year 1961-62.

Subsequently, Camilla has been designated as one of the Outstanding Fifty Women on campus. There are 7,000 women enrolled in the university. Last February she was selected as the outstanding Holstein 4-H Girl of New York, receiving the award at their state association meeting. She plans to do graduate study in genetics at M.S.U.

The other dairy senior who was especially recognized at the All-College convocation is KENT STEVENS. His grade point average of 3.82 is highest of all seniors, for which his name is engraved on the scholarship plaque. Kent also received the Borden Agricultural Scholarship Award of $300, the Farm Bureau award of $500, and his name is inscribed on the Alpha Zeta plaque. Subsequently, Kent has been selected to Phi Kappa Phi and has been named for a Purina Scholarship of $2,000 for graduate study. He will study at M.S.U. in the field of Dairy Physiology.

Massachusetts

Professor Emeritus J. H. Frandsen Marries

Mrs. Edna Wight Loewy of Orlando, Florida, and Julius Herman Frandsen of Orlando, Florida, and Amherst, Massachusetts, were married May 24.

The ceremony took place in the Unitarian Church in Orlando, Florida, with the Reverend John Fuller, pastor of the church, officiating.

Mrs. Frandsen was born in Spencer, Iowa, educated in New England, and is a graduate nurse. She spent 23 yr. as a public nurse with the Visiting Nursing Service of New York City. Landscaping, gardening, and house decorating are among her hobbies.

Professor Frandsen is Emeritus Professor of Dairy Industry of the University of Massachusetts, Amherst. In recent years he has been engaged in the preparation and publishing of a Dairy Handbook and Dictionary. He is also co-author of a new book, Ice Cream and Related Products, released June 14.

Dr. and Mrs. Frandsen are spending their honeymoon in New England, followed by a trip to Hawaii later this summer. They will be at their home at 92 High Street, Amherst, Massachusetts, after June 15, and at Orlando, Florida, 1510 E. Amelia Avenue, after October 15.

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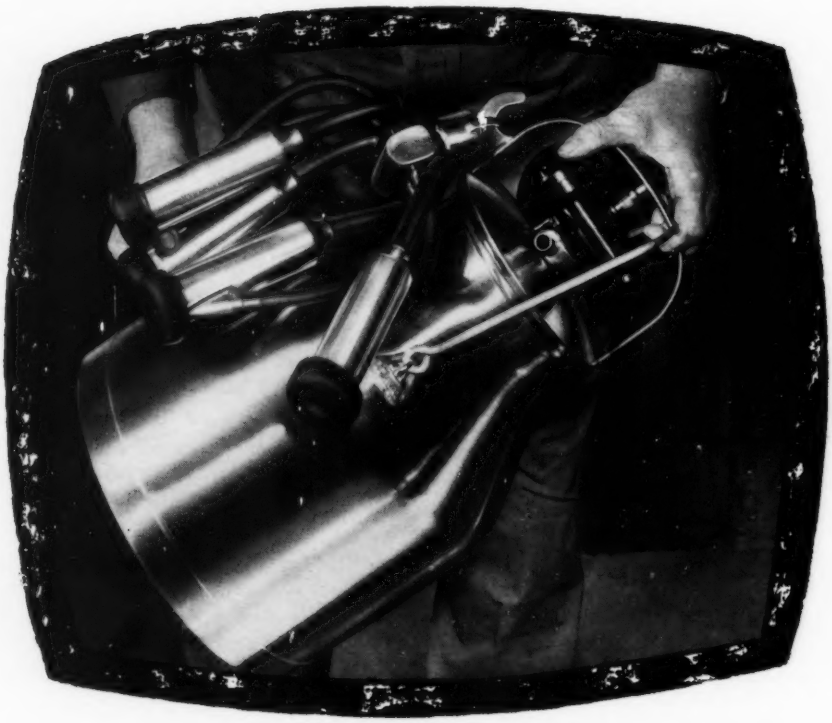

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Test #3: Hypochlorite and acid cleanser

B-K Powder with cleanser	9 mg.

Test #4: Measuring effect of water hardness when Hypochlorite is used with poor hard water cleanser

	Hardness	*Milkstone Deposit*
B-K Powder with cleanser	0	6 mg.
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New Jersey

A. C. Menge Joins Rutgers Staff

DR. A. C. MENGE will join the staff of the Rutgers University Dairy Research Farm, Sussex, July 1.

A. C. Menge

A specialist in animal physiology and reproduction, Dr. Menge will teach and do research work. He will take the place of DR. J. P. MIXNER, who will become chairman of the Department of Dairy Science in New Brunswick, July 1.

Dr. Menge recently received his Ph.D. in endocrinology from the University of Wisconsin. His thesis was, Studies on Embryonic Failure and Embryonic Mortality.

Born in 1934, in Harvard, Illinois, Dr. Menge was reared on a dairy farm. He attended Harvard (Illinois) Community High School. He did his undergraduate work at the University of Illinois and received a Master of Science degree in genetics from the University of Wisconsin in 1958.

West Virginia

The 10th Annual W.V.U. Student Dairy Cattle Congress was held this spring at the Dairy Pavilion. DEAN E. J. NESIUS, College of Agriculture, Forestry and Home Economics, crowned MISS LINDA WELSH, Adah, Pennsylvania, Queen of the Congress. Her attendants were MISS SANDRA VAN METER of Lewisburg, West Virginia, and MISS SUSAN STUART of Lost Creek, West Virginia. In the Showmanship contests, top winners for the various breeds were: Ayrshire, EDWARD SHEETS of Greenbank; Guernsey, MARY EDNA SMITH of Buckhannon; Holstein, TERRY NICHOLSON of Wolf Summit; and Jersey, STEVE HANNAH of Clarksburg. The H. O. Henderson Grand Champion Showman trophy was presented to Terry Nicholson by DR. I. D. PORTERFIELD, Chairman of the Department of Dairy Science.

The staff of the Dairy Science Department of the College of Agriculture recently conducted its 6th Judging School. The official judge was EDSEL GAINER, Assistant Extension Dairyman, West Virginia University. Classes of calves, heifers, and cows of the four major breeds were evaluated. During an evening session, DR. R. G. MITCHELL, chairman of the School, discussed Dairy Cattle Selection, and

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DR. I. D. PORTERFIELD, Chairman of the Dairy Department, led a discussion on Pedigree Evaluation. National breed club fieldmen, county agents, vo-ag instructors, and dairymen made up the 40 men participating in the School.

Delaware

New Personnel at University of Delaware Extension

The creation of the position of Vice-President for University Relations has been announced by the University of Delaware's board of trustees.

As Vice-President for University Relations, DR. G. M. WORRILOW will be responsible for external relations and activities, including the supervision and direction of the extension division, alumni, and public relations, and such other activities and functions as the President may assign. He also will have supervision over the urban services program being undertaken by the university with a 5-yr. grant from the Ford Foundation.

Dr. Worrilow will continue to serve as Dean of the School of Agriculture and will supervise the school in his dual capacity as Vice-President and Dean.

Vice-President Worrilow is a native of Cecil County, Maryland, and has served on the University of Delaware staff since 1927. In 1954 he was honored by his Alma Mater, the University of Maryland, with the degree of Doctor of Agriculture for leadership in his own state, and for distinguished and effective service in furthering national educational and research programs in the field of agriculture.

He was appointed Professor of Dairy Husbandry and Extension Specialist at the University of Delaware in 1941. He became Associate Director of the University's Agricultural Extension Service in 1943, and director of the Experiment Station and the Extension Service in 1948. In 1954, he was appointed Dean of the School of Agriculture in addition to the Post of Director.

Named Associate Dean of Agriculture was DR. MORRIS S. COVER, Chairman and Professor of Animal and Poultry Science. Dr. Cover is nationally known for his research in poultry diseases. He came to the University in 1952 and became department chairman in 1959. He teaches pathology and hygiene courses and has written numerous publications on poultry diseases.

A native of Harrisburg, Pa., Dr. Cover is a graduate of the University of Pennsylvania,

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where he earned his D.V.M. degree. After teaching briefly at the University of New Hampshire, he went to Kansas State College, where he earned his Master's degree while serving as an instructor in veterinary medicine. The Ph.D. was conferred at the University of Illinois in 1952.

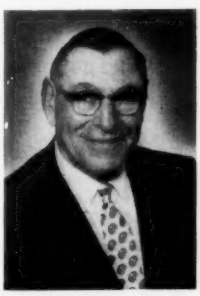
Promoted to associate director of the Delaware Extension Service in Agriculture and Home Economics was DR. S. M. GWINN, formerly assistant director. Dr. Gwinn has been with the Delaware Extension Service since 1948. He is a graduate of the University of West Virginia, where he received his Bachelor's degree in agricultural education in 1947 and his Master's degree in the same field in 1948. He became Sussex County 4-H Club agent in 1948.

In 1956, he received a Kellogg Foundation fellowship to the National Agricultural Extension Center for Advanced Study at the University of Wisconsin. After receiving his Ph.D. degree in cooperative extension administration in 1958, he returned to the University of Delaware to resume his position as state 4-H Club leader. In 1959, he was appointed assistant director of the Extension Service in Agriculture and Home Economics.

Pennsylvania

Robert Rosenbaum Honored

ROBERT ROSENBAUM of 8207 Cedar Rd., Elkins Park, Philadelphia, Pa., received the 1961 Humanity Award of the American Overseas Association, June 24, at the annual convention of the association in Miami Beach, Fla.



Robert Rosenbaum

The association's members are men and women who served overseas with the American Red Cross during World War II and the Korean conflict. Each year at its convention the group honors some individual who has performed outstanding services to humanity.

Rosenbaum was selected because of his efforts to secure better nutrition, sanitary conditions, and new industries in many underdeveloped countries. He first became interested in advancing better nutrition through improved diets in other nations when the Inter-American Committee for the Dairy In-

dustries was formed in 1941. Cuba, Jamaica, Haiti, the Dominican Republic, Puerto Rico, and the Virgin Islands are some of the places in which he has carried on his endeavors to improve pasteurization facilities and sanitary conditions on farms. He founded the Dairy Society International in 1946 and was official delegate for the U.S.A. at the International Dairy Congress in Rome in 1956.

Rosenbaum has been an active supporter of the National Students Dairy Products Judging Contest, which is sponsored each year by the American Dairy Science Association and Dairy Supplies Association.

INDUSTRY NEWS

Borden Forms International Division

Formation of an international division of the Borden Foods Company, and the appointment of J .V. BASSETT as executive vice-president of it, has been announced. The new division embraces the company's export and foreign operations. E. J. CRUGER, W. R. OLMSTEAD, and V. J. STOLFI, formerly assistant vice-presidents for foreign operations, have been named vice-presidents of the international division. N. J. DENAUT, director of overseas development, has been named assistant vice-president of the international division. The international division is responsible for sales of Borden's food and dairy products in over 100 countries and manufacturing operations in ten foreign nations.

Appointment of R. F. BUTTERFIELD as district manager of the Southern California district of The Borden Company's Western Division has been announced.

Mr. Butterfield joined Borden's in 1949 as a full-line salesman and supervisor in San Francisco. In 1952, he was transferred to the company's New York headquarters as assistant product manager, and subsequently became district sales manager for the grocery products division in Columbus and Cincinnati, Ohio.

Foremost Inaugurates Dairy Plant in the Philippines

An important addition to the dairy industry in the western Pacific area has recently been

ANIMAL PRODUCTION

Journal of the British Society of Animal Production

Contents of Volume 3, Number 2, June, 1961

BELLIS, D. B. AND TAYLOR, J. Nutritional factors in lean meat production of pigs.

CHAMBERS, D. T. Effect of two meal supplements on calves receiving autumn grass.

COSTAIN, R. A. AND MORGAN, J. T. Effect of the energy and protein status of the diet on productive efficiency in the pig.

CUNNINGHAM, J. M. M., EDWARDS, R. A. AND SIMPSON, M. Rearing lambs on a synthetic diet.

DUNIEC, H., KIELANOWSKI, J. AND OSINKA, ZOFIA. Heritability of chemical fat in the loin muscle of baconers.

GAHNE, BO. Transferrins in serum and milk of Swedish cattle.

HANCOCK, J. L. AND HOVELL, G. J. R. Effect of semen volume and sperm number on fertility of intra-uterine inseminations of pigs.

HARRINGTON, G. AND POMEROY, R. W. Yields of cuts from Wiltshire bacon sides related to length and carcass measurements.

LATHAM, J. O. AND ROGERS, C. Influence of breed and sex on carcass characters of beef cattle.

LODGE, G. A., MCDONALD, I. AND MACPHERSON, R. M. Relative influence of birth weight and creep-feed on 3- and 8-week weights of pigs.

LUCAS, I. A. M., LIVINGSTON, R. M. AND MCDONALD, I. Copper sulphate as a growth stimulant for pigs: effect of level and purity.

SHORTHOSE, W. R. AND LAMMING, G. E. Effect of different oestrogens and oestradiol plus progesterone on growth and carcass of hoggets.

WHITELAW, F. A., PRESTON, T. R. AND NDUMBE, R. D. Nutrition of the early-weaned calf (I).

WHITELAW, F. A., PRESTON, T. R. AND DAWSON, A. S. Nutrition of the early-weaned calf (II).

This journal is a continuation and expansion of the *Proceedings of the British Society of Animal Production* (1944-58), and has appeared in volumes of two parts but in order to make provision for the increasing flow of material submitted for publication **it will in future, commencing with Volumes 3 (1961), appear in annual volumes of three parts** in February, June and October. **The annual subscription will be 45s. ($7.50 in U.S.A. and Canada),** single parts 17s. 6d. ($2.75 in U.S.A. and Canada) net. Orders and subscriptions should be sent to:—

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J. E. WICKERSHAM, who heads Foremost's international operations and is President of International Dairy Engineering Co., Inc., a Foremost subsidiary, stated that Foremost often operates overseas by providing local businessmen with technical and engineering assistance.

Y. S. KWONG, vice-president of the new Philippine company, also expressed the opinion that the establishment of the Manila Golden State Plant would bring better nutrition to Philippine consumers, while making a major contribution to the economy of the islands.

The opening of the 40,000-square foot plant, located in Makati, a new industrial district of Manila, was attended by many prominent citizens of the Philippines, including PRESIDENT C. P. GARCIA, chief executive of the Islands.

Chas. Pfizer and Company Acquires New Subsidiary

PAUL HALMBACHER, president of Paul-Lewis Laboratories, Inc., and J. E. MCKEEN, president of the Chas. Pfizer & Co., Inc., have announced the transfer of the capital stock of Paul-Lewis to Pfizer. Mr. Halmbacher, who will continue as president, said that Paul-Lewis will operate as an independent subsidiary with no staff or administrative changes. CARL DEDERICH, sales manager of the Paul-Lewis dairy division, stated that this will enable Paul-Lewis to broaden its scope of products and research for the dairy industry. The Charles Pfizer Company, established in 1849, has grown into one of the leading manufacturers of fermentation, chemical, and agricultural products.

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M. Hales Joins Paul-Lewis Laboratories

MIKE HALES has accepted the position of Director of the Dairy Division for the Paul-Lewis Laboratories. Mr. Hales will have operational responsibility for the complete division and direct its present expansion program of products and services for the dairy industry.

Mike Hales

Mr. Hales is a graduate of the University of Wisconsin in dairy industry and holds a Master's Degree from the University of Missouri. Since leaving school, his time and efforts have been devoted to the development of enzyme preparations, food colors, and cultures for the dairy industry, a field in which he is recognized as an international authority. He has worked in close liaison with many researchers at academic institutions and has been largely responsible for the practical application of many of their findings. His numerous papers on the many facets of cottage cheese manufacture have helped the dairy industry.

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Dairy Guild of America Appoints Howard Levine

Howard Levine

The Dairy Guild of America, a group of united independent leading dairymen, has appointed HOWARD LEVINE as their Membership Director. Mr. Levine has a rich background in sales and promotional work and is well-qualified for his present challenging duties.

He is presently engaged in interviewing dairies in all sections of the country for membership in the Dairy Guild.

Available for member use are the services of the Dairy Research Bureau. The Bureau's laboratory facilities and technical staff are among the finest in the industry. Not only are new products being developed for the exclusive use of Dairy Guild members, but a field staff of technicians is available to Guild members to help them improve their operation and to solve technical problems that arise occasionally in any dairy. Guild members who have used this service have commented enthusiastically on this phase of the program.

Specifically, the Guild assists in advertising and sales promotion, group purchasing, management, and packaging problems. Inquiries are invited from those dairymen who would like to compete more profitably, and who feel they can qualify for membership in the Dairy Guild of America. Address: Dairy Guild of America, P. O. Box 3-G, Cincinnati 3, Ohio.

Sanitarians Select Des Moines, Iowa for 50th Meeting

The Hotel Savery in Des Moines, Iowa, will be the site of the 50th anniversary meeting of International Association of Milk and Food Sanitarians, August 14–17, 1961. The Iowa Association of Milk Sanitarians will be host group to the other affiliates of the International. A local committee headed by R. L. SANDERS, of the Des Moines City Health Department, is making plans to welcome the nationwide group of sanitarians who normally attend the annual conventions.

Pasteurization Training Course

The Training Program of the Robert A. Taft Sanitary Engineering Center announces

a training course on Milk Pasteurization Controls and Tests (95M) scheduled for presentation in Cincinnati, Ohio, September 12–14, 1961.

Applications or requests for information should be addressed to the Chief, Training Program, Robert A. Taft Sanitary Engineering Center, 4676 Columbia Parkway, Cincinnati 26, Ohio, or to a PHS Regional Office Director.

THESES COMPLETED

M.S. Degree:

R. M. Cook—Ruminal absorption of acidic and neutral amino acids. University of Illinois.

R. V. Johnson—Influence of month of calving on lactation milk yield, University of Illinois.

David Clyde Martin—A study of breeding efficiency in dairy cattle. West Virginia University.

Dayal D. Shrivastava—Utilization of sesame and peanut oil by young dairy calves. University of Illinois.

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RESEARCH PAPERS

NATURAL STATE OF MILK PROTEINS. I. COMPOSITION OF THE MICELLAR AND SOLUBLE CASEIN OF MILK AFTER ULTRACENTRIFUGAL SEDIMENTATION

H. U. BOHREN AND V. R. WENNER
Nestlé Products, Research Laboratories, Vevey, Switzerland

SUMMARY

A technique is described for the separation by ultracentrifugation of up to 99% casein from milk. The casein gel is transformed by an ion exchange procedure to colloidal alkali caseinate and finally submitted to electrophoretic analysis. This technique guarantees that there is no loss in casein fractions.

The level of the residual casein soluble in the ultracentrifugal whey is influenced by the temperature of centrifugation and by added calcium. Temperatures above 20° C. or added Ca up to 6 m*M* per 100 ml. of milk before centrifugation improve the casein sedimentation considerably.

If no Ca is added to the milk centrifuged at 20 or at 4° C., the amount of soluble casein left is about 6 or 15%, respectively. The soluble casein shows a relatively much higher percentage of β- and γ-casein compared to whole casein.

The electrophoretic behavior of casein has been examined by many research workers who have used mostly the Warner (12) method to prepare casein samples. It is known that by this method a considerable amount of γ-casein is lost through repeated washings. Furthermore, it is necessary to extract the casein with organic solvents to eliminate fat, since lipid material at a level of more than 0.1% in the solution disturbs the patterns of free electrophoresis, as pointed out by Tobias *et al.* (11).

The advantage of the separation in the ultracentrifuge is that casein can be prepared in its initial state and, if centrifuged from raw milk, the amount of lipid material in the sediment is far below the limit where an interference in the electrophoretic analysis has to be expected. However, the casein depletion is not quantitative. It is a well-known phenomenon that casein in micellar form is in equilibrium with soluble casein as observed, for example, by von Hippel and Waugh (5) or by Ford *et al.* (2).

In the present study, the technique of the casein separation from milk in the ultracentrifuge is described in emphasizing the factors affecting the amount and composition of the soluble casein. The gel-like casein sediments have been transformed into a clear colloidal solution of sodium caseinate by a simple ion exchange treatment. Results are given on the P/N-ratios of the isolated caseins and their characteristics on electrophoretic analyses.

METHODS

Casein separation in the ultracentrifuge. Casein was isolated from pooled skimmilk. In some cases, pooled whole milk was used for comparative experiments.

Received for publication March 11, 1960.

Centrifugation was effected in a Spinco Model L preparative centrifuge. In skimmilk (or whole milk), the casein depletion reached a constant minimum value after 180 min. at 21,000 r.p.m. (50,000 times gravity) and 20° C. or 300 min. at 21,000 r.p.m. (50,000 times gravity) and 4° C.

The supernatant whey containing the residual soluble casein was carefully decanted from the sediment. Two milliliters of 3.0 *M* $CaCl_2$ were added in stirring to 100 ml. of centrifugal whey. After a second centrifugation for 180 min. at 21,000 r.p.m. and 20° C., the soluble casein was quantitatively precipitated and was recoverable as a rather compact gel.

The amount of residual soluble casein can be reduced to a minimum level of only about 1% by the following technique: To 100 ml. of pooled milk, 2 ml. of 3.0 *M* $CaCl_2$ are added in drops in stirring, followed by a neutralization to pH 6.7 with 1.3 to 1.5 ml. of 1.0 *N* KOH. Centrifugation is effected at 20° C. and at 21,000 r.p.m. (50,000 times gravity) for 180 min. or 40,000 r.p.m. (100,000 times gravity) for 30 min.

Preparation of the colloidal Na-caseinate-solution. The casein gel in the tubes was gently rinsed with a few milliliters of distilled water, weighed, then squeezed through a 625-mesh/sq cm sieve and the volume made up to 10 ml. per gram casein sediment with distilled water. The Ca was exchanged against Na at a pH between 6.2 and 6.7 by means of a Na-H-cation exchange resin prepared as follows: To 16 ml. of Na-saturated Amberlite IR 120 add 2.4 ml. of 1 *N* HCl. Stir for 5 min. and let stand for about 2 hr. Rinse four times with 50 ml. distilled water by decantation and finally eliminate the water by suction on a Büchner funnel.

The Na-H-Amberlite was then added to the casein suspension in stirring. After 40 to 50 min. of exchange time, about 95% of the Ca was replaced by Na. The colloidal solution of about 2% Na-caseinate was decanted from the Amberlite, cooled in ice-water, and centrifuged at 15,000 r.p.m. for 20 min., to eliminate residual resin by sedimentation and any denatured serum proteins present. After centrifugation, the clear supernatant solution was cautiously poured off. The sediment and the supernatant thin layer of lipid material were discarded.

Electrophoresis was carried out in an Antweiler-microelectrophoresis apparatus (13), equipped with a Philpot-Svensson optical system for the schlieren analysis, and with an optical table for interferometric measurements to determine the accurate protein concentration.

The U-tube of the Tiselius-type cell has a length of 8.5 cm., with a cross section area of 0.05 sq. cm. The volume of the protein solution is 0.25 ml. Protein boundaries migrate over a distance of 0.15 to 0.5 cm.

During electrophoresis, the current is maintained at a constant value of 1.8 m amp., with a resulting voltage of 75 to 85 v.

All quantitative electrophoretic measurements have been made in the ascending channel of the cell, as only the rising boundaries can be reproduced with sufficient distinctness for interferometric readings. These readings are then mechanically integrated to obtain the electrophoretic diagram where the areas of the separated peaks are represented as percentages of total protein.

The casein solutions used contained 1.0 to 1.3% protein and were dialyzed against the 50-fold volume of a barbital-acetate buffer at pH 7.4 (6).

Nitrogen and phosphorus analysis. To eliminate traces of solid calcium phosphate and phospholipids in soluble casein, 0.5 ml. of 10% acetic acid were added in stirring to 5 ml. of the Na-caseinate solution and the suspension was dialyzed at 4° C., first against 250 ml. of a Sörensen citrate buffer at pH 5.0 for 14 hr., then against distilled water for 24 hr. The water was changed twice. The dialyzed casein suspension was slowly added to a sixfold volume of a 4:1 mixture of alcohol (96%) and carbon tetrachloride at a temperature of 28° C. The mixture was let stand overnight in the dark at room temperature. The precipitate and solution were separated by decantation and filtration. The residue was washed on the filter with three times 15 ml. alcohol–carbon tetrachloride mixture and then with three times 15 ml. ether. Finally, the casein was dried for 20 hr. in vacuo over concentrated H_2SO_4. Nitrogen determinations were made by a micro-Kjeldahl method. Phosphorus was analyzed according to the method of Fiske and Subbarow (1).

RESULTS AND DISCUSSION

Sedimentation of casein in the ultracentrifuge: Factors affecting the level and composition of the residual, soluble casein. We have examined the possible degree of maximum casein depletion at 4 and 20° C. by centrifuging skimmilk at 50,000 times gravity. The residual nonseparated casein in the centrifugal whey is hereafter called soluble casein. Levels of the soluble casein related to different temperatures and rotor speeds are given in Table 1. The level of

TABLE 1

Levels of residual, soluble casein in whey after ultracentrifugation of milk

No. times gravity	Temperature	Centrifugation time [a]	Soluble casein in supernatant whey [b]
	(° C.)	*(min.)*	*(%)*
50,000	4	280	10–15
50,000	20	180	3–7
100,000	4	50	10–15
100,000	20	30	3–7

[a] Centrifugation time necessary to reach the flat part of the sedimentation curve.
[b] The soluble casein is given in per cent of the casein initially present in the milk (= 100%), determined by the Rowland method (9).

soluble casein for a given rotor speed and temperature varies from one milk to another. This phenomenon has not been investigated. At any rate, it is evident that (for a given milk) the degree of sedimentation of casein in the ultracentrifuge depends on the temperature of centrifugation. Zittle and Pepper (15) have reported on the important temperature coefficients of casein aggregation. The better solubility of casein at low temperatures might be due to a

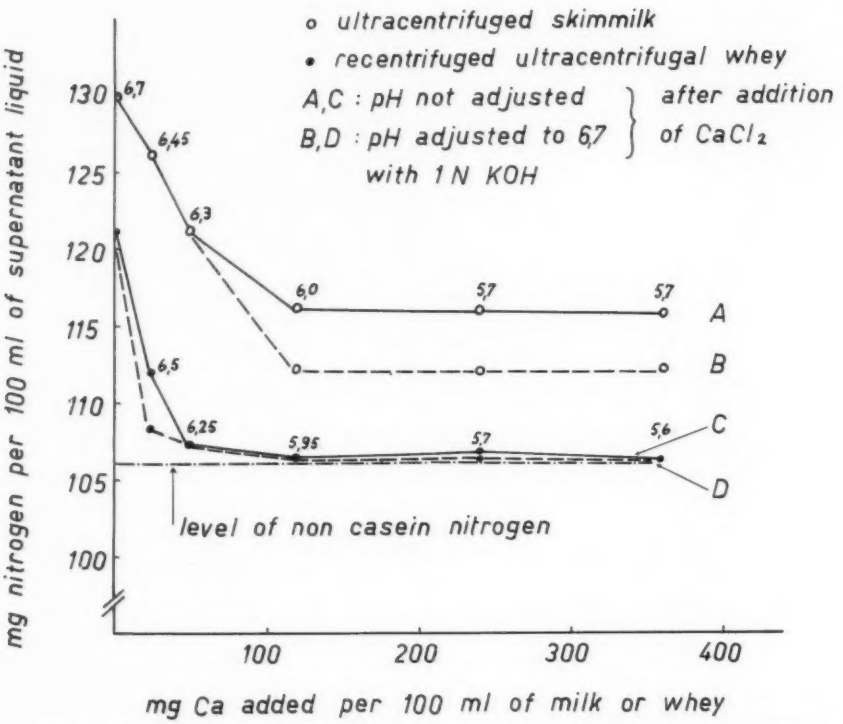

FIG. 1. Levels of residual, soluble casein in ultracentrifuged skimmilk or recentrifuged ultracentrifugal whey as a function of added calcium. Ultracentrifugation at 21,000 r.p.m. and 20° C. for 180 min.

higher hydration at the phosphate groups of casein, as suggested by Zittle *et al.* (14).

Addition of Ca to milk lowers the level of residual soluble casein, as shown by the Curves A and B of Figure 1 (after centrifugation at 21,000 r.p.m., 20° C. for 180 min.). Here, it is the excess of calcium which reduces the dissociation of the calcium-phosphatase-casein complex, favoring the micellar state of the casein and, therefore, allowing for a more quantitative sedimentation. pH adjustment to 6.7 by KOH still improves this effect. It is, however, not possible, even with a large excess of added Ca, to obtain from milk a casein-free ultracentrifugal whey. The minimum level of residual casein is at about 1% of the initial amount as determined by the Rowland (9) method.

If Ca is not added to milk but only to the centrifugal whey containing the soluble casein, and if a second centrifugation is effected at 20° C. (21,000 r.p.m., 180 min.), casein can be completely eliminated from the whey without any risk of precipitating the whey proteins, as demonstrated by the Curves C and D of Figure 1.

The P/N ratios in Table 2 indicate that soluble casein and whole casein differ in composition.

We have examined the residual casein in the supernatant liquid of different portions of a milk sample, each portion being centrifuged at 20° C. for a longer time than the preceding one. The N/P ratios are found to be the same as in whole casein at levels of 15% or more residual casein. Therefore, the different composition of the residual, finally soluble casein only becomes evident at levels below 15%.

TABLE 2

P/N relations of residual casein

Symbol of preparation	Isolated from [a]	Amount in per cent of total casein [b]	P/N ratio [c]
SC 20	Whey of skimmilk, centrifuged at 20° C. for 180 min.	5.7	0.045
SC 4	Whey of skimmilk, centrifuged at 4° C. for 300 min.	15	0.044
RC 20	Whey of skimmilk, centrifuged at 20° C. for 120 min.	15	0.054
CC	Skimmilk (+ added Ca and neutralized) centrifuged at 25° C. for 180 min.	99	0.055

[a] The skimmilk as starting material was the same for samples SC 20, RC 20, and CC. Centrifugation was effected at 21,000 r.p.m. (50,000 times gravity).

[b] Amount of casein isolated from whey or skimmilk, given as percentage of total casein present in skimmilk.

[c] P/N ratios of: whole casein = 0.055; α-casein = 0.064; β-casein = 0.04; γ-casein = 0.007, according to Gordon *et al.* (3, 4).

The electrophoretic patterns and the composition of whole and soluble casein, as given in Figures 3 and 4 and in Table 3, respectively, will be discussed later.

Preparation of the colloidal Na-caseinate. As indicated under Methods, the Ca of the ultracentrifugal casein can be replaced by Na in the pH range of 6.2 to 6.7 by means of a cation exchange resin. If the exchange is effected with a resin saturated with Na, the pH follows the Curve A of Figure 2. By using Na-Amberlite containing a certain amount of H-equivalents, the alkaline reaction can be avoided. Any risks of protein denaturation are avoided and, moreover, the Ca exchange is favored in the acid pH range, as shown by residual Ca analysis in Figure 2. The Na-H-Amberlite used for our caseinate preparations produces the pH variations shown by Curve D. Further acid exchangers should not be used, in order to avoid any casein precipitation during the pronounced pH shift at the beginning of the exchange. The residual Ca is elimi-

TABLE 3

Relative areas of the electrophoretic components of whole casein and soluble casein (see Figures 3 and 4)

Preparation [a, b]	Component No.	Fraction	Relative area per cent
Whole casein (CC)	1	α_I	59.5
	2	α_{II}	8.5
	3	β	24.7
	4	γ	4.3
	5	x	3.0
Soluble casein (SC 20)	1	α	37.2
	2	β	43.0
	3	γ	15.4
	4	x	4.4
Soluble casein (SC 4)	1	α	30.0
	2	β	57.2
	3	γ	12.8

[a] Symbols, see Table 2.
[b] Electrophoretic diagrams, see Figures 3 and 4.

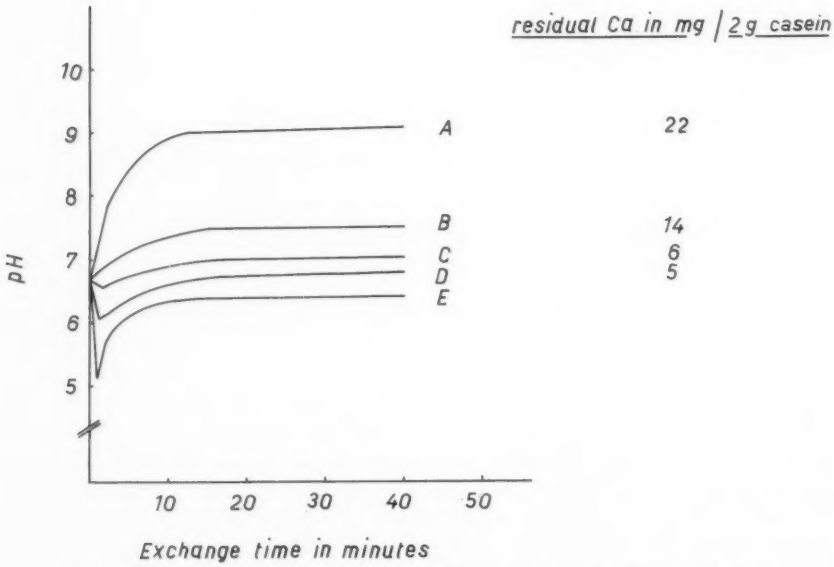

FIG. 2. pH changes in 100 ml. of 2% suspensions of ultracentrifuged casein as a function of calcium-sodium ion exchange with 32 meq. Amberlite IR 120, as a mixture of:

A: 32.0 meq. Na^+ + 0.0 meq. H^+
B: 31.2 meq. Na^+ + 0.8 meq. H^+
C: 30.0 meq. Na^+ + 2.0 meq. H^+
D: 29.6 meq. Na^+ + 2.4 meq. H^+
E: 28.8 meq. Na^+ + 3.2 meq. H^+

nated during the buffer dialysis and will, therefore, not disturb in any way the electrophoretic analysis.

Electrophoretic analysis of casein. The reliability of our electrophoretic method of quantitative protein analysis has been checked by running calibration trials with purified α-, β-, and γ-casein fractions. The protein concentration determined interferometrically was in agreement with the Kjeldahl values for every casein fraction.

Mixtures of α-, β-, and γ-casein of a known composition have been separated electrophoretically. The recovered amounts of α- and β-casein are in agreement with the calculated values. On the other hand, only about 50% of the γ-casein could be recovered. This loss must be due to complex forming with α- and β-casein. With a sufficiently high relative percentage of γ-casein in the mixture, the increase in α- and β-casein due to complex forming with γ-casein could be observed. These complexes have no distinctively different mobilities compared to the pure α- or β-casein.

We suppose that it is for the same reason that the separation of γ-casein from the natural whole casein in the free electrophoresis is not quantitative and figures given in the literature for the amount of γ-casein present in whole casein might be too low. MacRae and Baker (7), for example, found by paper electrophoresis a percentage composition of the casein components which is higher than the 3% quoted by other authors.

The electrophoretic patterns of whole casein are shown in Figure 3, and the results of the quantitative evaluation, based on measuring the relative areas after mechanical integration of the interferometric readings, are presented in Table 3. The figures in Table 3 are in conformity with the results of whole casein composition given in the literature. The relative amount of 4.3% of γ-casein found has to be considered as about 100% too low, mainly for the reasons of complex forming, since loss in γ-casein due to preparation can be excluded with our method. The possible contamination of casein with whey proteins falls within the limits of error of the method.

The composition of soluble casein, as given in Figure 4 and Table 3, differs widely from that of whole casein. We have checked the composition of soluble casein by adding different amounts of analyzed whole casein and we have found that the fast-moving component of soluble casein has the same mobility as the α-fraction of whole casein. It is certainly possible that the α-like fraction of the soluble casein contains mainly the Ca-insensitive kappa-casein, which was found by McGann and Pyne (8) and by Sullivan *et al.* (10) to be present in relatively high amounts in the smallest casein micelles and in soluble casein.

Besides this α-like fraction, we have found in soluble casein considerable amounts of β- and γ-casein. Starting from the recognized fact that β- and γ-casein, especially at lower temperatures, have less tendency to form micelles than α-casein, these differences in the relative amount of α-, β-, and γ-casein in soluble casein compared to whole casein might be explained as follows:

As long as β- and γ-casein are combined in a micelle with an excess of Ca-sensitive α-casein, they can be centrifuged. With progressing casein deple-

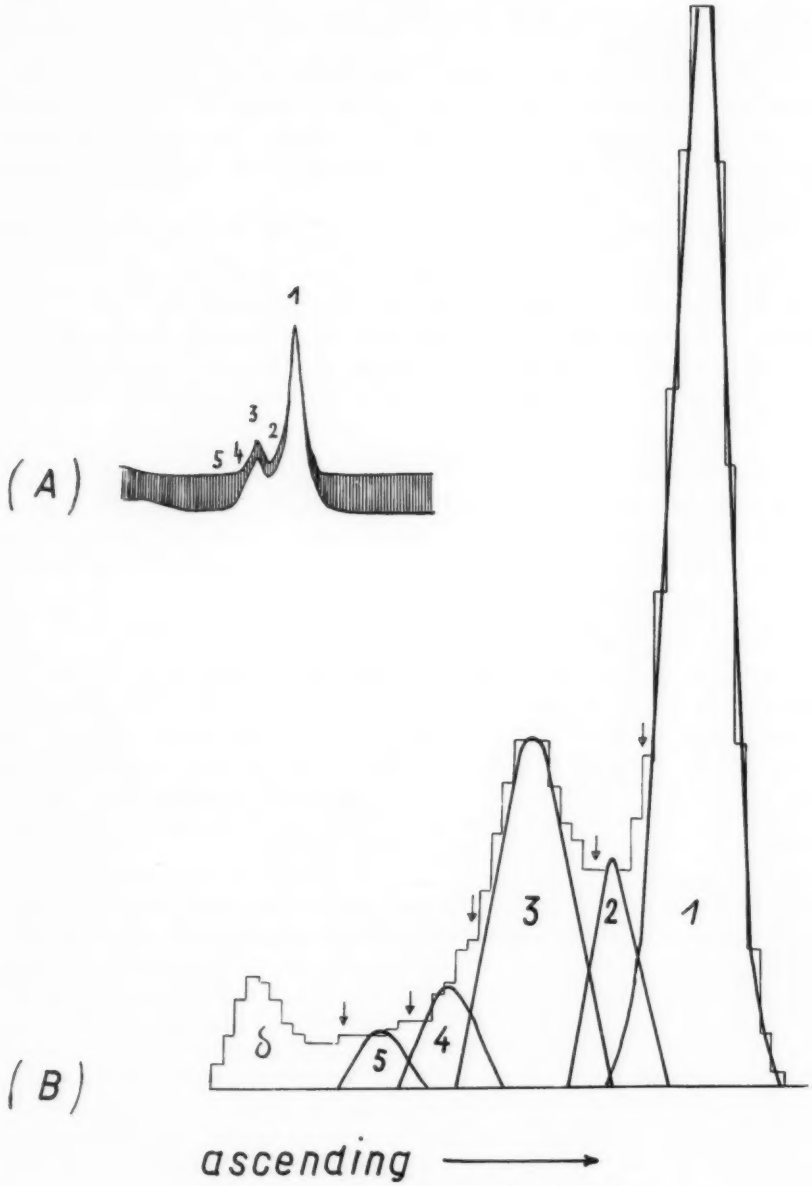

FIG. 3. Electrophoretic diagram of whole casein.

A: Reproduction of the schlieren photograph after 9 min. run of electrophoresis.
B: Diagram obtained from integrating the interferometric measurements. The distances of the fractions along the horizontal axis have no correlation with their mobilities.

tion, the residual casein contains relatively more and more of the Ca-insensitive kappa-fraction, which is found especially in the smallest micelles (8). Finally, β- and γ-casein remain in solution together with the kappa-fraction and only the less soluble Ca-sensitive α-casein continues to precipitate. The result is a soluble casein, poor in α-casein (see Table 3 and Figure 4).

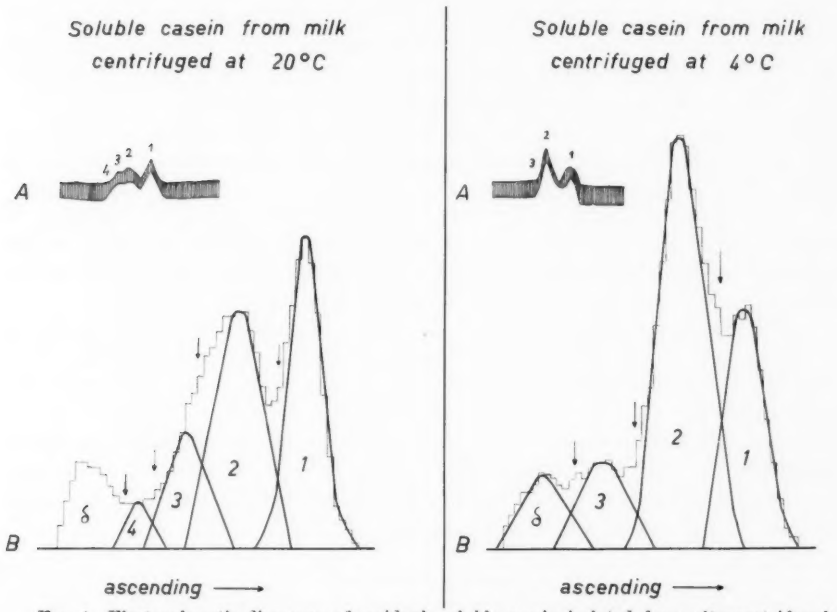

FIG. 4. Electrophoretic diagrams of residual, soluble casein isolated from ultracentrifugal whey.

A: Reproduction of the schlieren photograph after 9 min. of electrophoresis.

B: Diagram obtained from integrating the interferometric measurements. The distances of the fractions along the horizontal axis have no correlation with their mobilities.

If milk is centrifuged at 20° C., the level where solubilization occurs and the different composition of the residual casein becomes obvious must be below 15% residual casein, since at 15% the P/N ratio and the electrophoretic patterns are still the same as for whole casein. The critical level might be at about 10% of residual casein, where Sullivan *et al.* (10) found the relative percentage of kappa-casein started to increase rapidly.

If milk is centrifuged at 4° C., about 15% of the casein becomes soluble and it may be noticed that it is richer in β- and poorer in α-like casein than soluble casein obtained at 20° C. (see Table 3 and Figure 4). In view of the better solubility of β-casein at lower temperatures, less kappa-casein is needed to prevent sedimentation and, therefore, higher levels of soluble casein with a higher relative percentage of β-casein are found.

A few experiments made on the composition of soluble casein isolated from pasteurized milk have shown that even moderate heat treatment induces a clear change in the composition of soluble casein. The results of these experiments will be published later.

CONCLUSIONS

The separation of casein from milk in the ultracentrifuge is considerably improved if Ca is added to the milk before centrifugation. At a level of 6 m*M* $CaCl_2$ per 100 ml. of milk, and after adjusting the pH to 6.7, up to 99% of casein have been separated at 20° C. and 50,000 times gravity in 3 hr. Higher Ca levels do not reduce the residual soluble casein any further.

The casein sediment has been prepared for electrophoretic analysis by an ion exchange procedure without any possible loss of protein, especially the γ-fraction.

Without $CaCl_2$ added to the milk, casein depletion was found to be 93 to 97% at 20° C. or 85 to 90% at +4° C. The remaining soluble casein in the supernatant ultracentrifugal whey can be separated quantitatively by a second centrifugation after the addition of 6 m*M* $CaCl_2$ to 100 ml. of whey.

Electrophoretic analysis showed the same components in soluble casein as in whole casein, but with a much higher percentage of β- and γ-casein. It is considered that the kappa-casein might be an important factor for solubilizing β- and γ-casein.

Ultracentrifuged whole casein, precipitated from milk at temperatures in between 4 to 20° C., is certainly deficient in β- and γ-casein. This deficiency can be rectified by adding Ca to the milk before centrifugation or by centrifuging the milk at a temperature of at least 40° C.

ACKNOWLEDGMENT

The authors wish to thank Professor B. E. Baker, McGill University, Quebec, Canada, for donating samples of α-, β-, and γ-casein.

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CHARACTERISTICS OF SEVERAL MINOR-PROTEIN FRACTIONS ISOLATED FROM BOVINE MILK [1]

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SUMMARY

Four minor-protein fractions were isolated from the same source of skimmilk by methods similar to those reported by Rowland for proteose-peptone, by Aschaffenberg for σ-proteose, by Jenness for milk component 5, and by Weinstein for the minor-protein fraction. A fifth fraction, the soluble membrane-protein, a constituent of the fat/plasma interfacial layer, was isolated from washed cream.

These fractions were characteristically low in nitrogen (10-14%), high in ash (3-7%) and phosphorus (0.6-1.5%), and contained hexose sugars. Free-boundary electrophoretic patterns demonstrated variable multicomponent systems containing what appeared to be a common major component. Sedimentation velocity diagrams of the skimmilk fractions revealed two apparently common boundaries, whereas the diagrams for soluble membrane-proteins showed more heterogeneity, suggesting that the constituent components differed from those of the skimmilk proteose-peptones.

The proteose-peptone, σ-proteose, and minor-protein fractions were rennin substrates. Heating (90° C.-30 min.) 1% solutions of milk component 5 concentrate and the soluble membrane-protein, two fractions obtained from raw milk without employing a heat-treatment step in the isolation procedure, produced changes in both the electrophoretic and sedimentation patterns. Further, heated milk component 5 concentrate was more susceptible to the action of rennin than its unheated counterpart. The membrane fraction was unaffected by rennin.

The classical proteose-peptone fraction, precipitated by 10% TCA but not by heating to 95° C. for 30 min., and the fat-globule membrane constitute the source of the minor-protein fractions of milk. These are complex protein systems quite different from the principal proteins of milk.

Rowland (7) classified the proteins in the fraction not precipitated by heating milk to 95° C. for 30 min. but precipitated by 10% TCA as proteose-peptone. Aschaffenburg (1) isolated a protein fraction from acid or rennet whey by salting out the heat-cleared sera with $(NH_4)_2SO_4$, which he called σ-proteose because of its pronounced surface activity. The fraction contained three electrophoretic peaks when examined in phosphate buffer at pH 8.0, and two ultracentrifugal boundaries in phosphate buffer at pH 8.6. Although Aschaffenburg presented data to establish the similarities between acid and rennet whey in respect to σ-proteose, he did not indicate clearly which preparation was characterized. Weinstein *et al.* (9) isolated a protein from heated, rennet whey by a scheme quite similar to that of Aschaffenburg's, which they called the minor-

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protein fraction. Two or three poorly defined electrophoretic peaks were reported (10). Larson and Rolleri (5) presented electrophoretic patterns of the proteins constituting the heat-stable residue of acid whey. Three electrophoretic peaks were noted, which were designated as Components No. 8, 5, and 3, in descending order of mobility, Component 5 occurring in the highest concentration. More recently, Jenness (4) obtained a fraction rich in whey component 5 from unheated skimmilk. Casein and the minor fraction were salted out of skimmilk saturated with NaCl. A component 5-rich fraction has been isolated in our laboratory by salting out with NaCl (saturated) from neutralized, acid whey.

Herald and Brunner (3) prepared two protein fractions from fat-globule membrane of normal cow's milk, which were designated as water-soluble and insoluble. The soluble fraction possesses heat stability and compositional characteristics quite similar to the classical proteose-peptone fraction, which suggests a possible relationship between the proteose-peptone fraction and the fat-globule membrane protein. Additional comparisons are indicated.

This study was initiated for the purpose of elucidating the chemical and physical characteristics of representative minor-protein fractions prepared from the same source of milk.

EXPERIMENTAL PROCEDURE

Preparation of the minor-protein fractions. Protein fractions were isolated from skimmilk and/or cream from pooled, unpasteurized milk obtained from the Michigan State University dairy plant during the winter of 1959-1960.

Proteose-peptone. A proteose-peptone fraction was obtained by following the fractionation principles used in Rowland's nitrogen distribution method. Casein and the associated heat-denatured whey proteins were precipitated with 1 *N* HCl at pH 4.6. The clear supernatant was neutralized, dialyzed free of salt, pervaporated, and lyophilized (Figure 1).

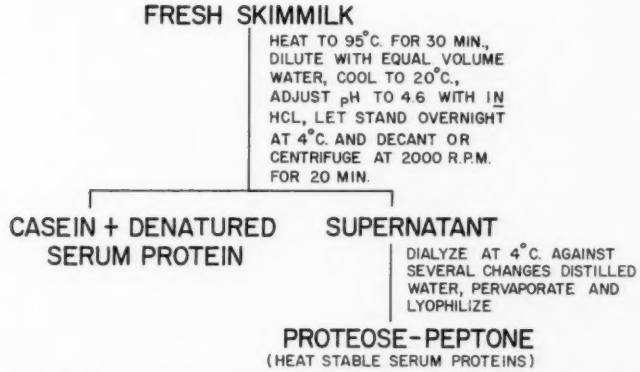
FRESH SKIMMILK
HEAT TO 95°C. FOR 30 MIN., DILUTE WITH EQUAL VOLUME WATER, COOL TO 20°C., ADJUST pH TO 4.6 WITH 1N HCL, LET STAND OVERNIGHT AT 4°C. AND DECANT OR CENTRIFUGE AT 2000 R.P.M. FOR 20 MIN.
CASEIN + DENATURED SERUM PROTEIN
SUPERNATANT
DIALYZE AT 4°C. AGAINST SEVERAL CHANGES DISTILLED WATER, PERVAPORATE AND LYOPHILIZE
PROTEOSE-PEPTONE
(HEAT STABLE SERUM PROTEINS)

FIG. 1. Schematic diagram showing the isolation of the proteose-peptone fraction based upon the principles of Rowland's nitrogen distribution scheme (7).

σ-proteose. This fraction was prepared by following the procedures employed in preparing the proteose-peptone fraction to the concentration step. At this point, the pervaporated solution was adjusted to half-saturation with $(NH_4)_2SO_4$. The flocculated protein was collected by centrifugation, washed with half-saturated $(NH_4)_2SO_4$ solution, slurried with water, dialyzed free of salt, and lyophilized (Figure 2).

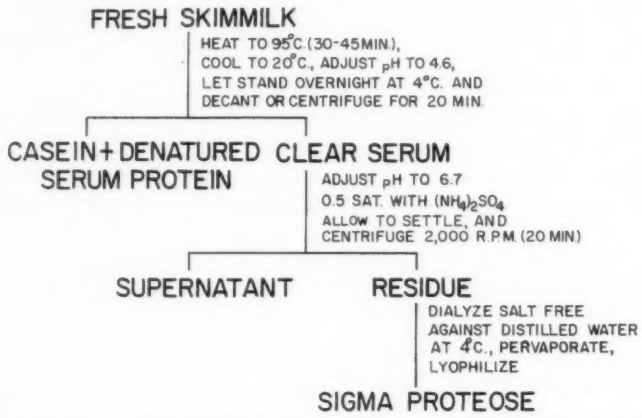

FIG. 2. Schematic diagram showing the isolation of the σ-proteose fraction (1).

The minor-protein fraction. Weinstein isolated the fraction from rennet whey by a method quite similar to that of Aschaffenburg's for σ-proteose. Raw skimmilk was warmed to 25° C., set with sufficient diluted rennet extract to form a clot in 15-20 min. The clot was cut into half-inch cubes and the whey expelled by warming to 50° C. The filtered whey was heated to 95° C. for 30 min., followed by cooling to room temperature. The heat-denatured proteins were removed by adjusting the pH to 5.5 and allowing the floc to settle. Weinstein employed high-speed centrifugation to clear the heated sera. The clear supernatant was neutralized, concentrated by pervaporation, and half-saturated with $(NH_4)_2SO_4$. The floc was collected, washed with half-saturated $(NH_4)_2SO_4$ solution, slurried with water, dialyzed free of salt, and lyophilized (Figure 3).

Milk component 5 concentrate. Essentially, the procedure as outlined by Jenness was followed. Raw skimmilk was saturated with NaCl and allowed to stand overnight at room temperature. The salted-out protein (casein + the minor fraction) was concentrated by centrifugation and washed 4-5 × with saturated salt solution to remove unaffected whey proteins. The washed protein mixture was slurried in water at pH 8.0 and dialyzed free of salt. Casein was precipitated with 1 *N* HCl at pH 4.6. The mucoidal-appearing supernatant was dialyzed, concentrated by pervaporation, and passed over an 1RC-50(H^+) resin column. The eluate, rich in milk component 5, was dialyzed, concentrated, and lyophilized (Figure 4).

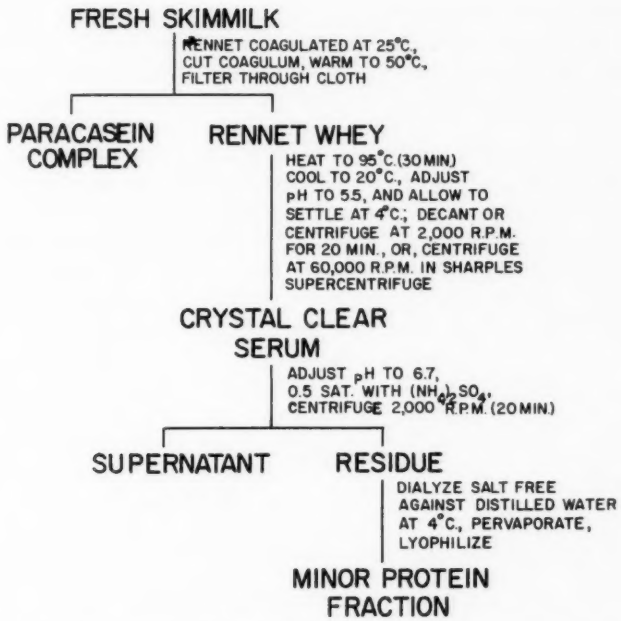
FRESH SKIMMILK
RENNET COAGULATED AT 25°C., CUT COAGULUM, WARM TO 50°C., FILTER THROUGH CLOTH
PARACASEIN COMPLEX
RENNET WHEY
HEAT TO 95°C.(30 MIN) COOL TO 20°C., ADJUST pH TO 5.5, AND ALLOW TO SETTLE AT 4°C.; DECANT OR CENTRIFUGE AT 2,000 R.P.M. FOR 20 MIN., OR, CENTRIFUGE AT 60,000 R.P.M. IN SHARPLES SUPERCENTRIFUGE
CRYSTAL CLEAR SERUM
ADJUST pH TO 6.7, 0.5 SAT. WITH $(NH_4)_2SO_4$, CENTRIFUGE 2,000 R.P.M. (20 MIN.)
SUPERNATANT
RESIDUE
DIALYZE SALT FREE AGAINST DISTILLED WATER AT 4°C., PERVAPORATE, LYOPHILIZE
MINOR PROTEIN FRACTION

FIG. 3. Schematic diagrams showing the isolation of the minor-protein fraction (9).

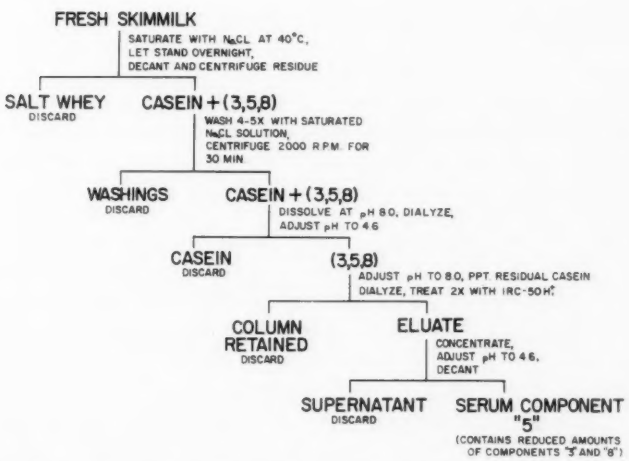

FIG. 4. Schematic diagram showing the isolation of milk component 5 concentrate based upon the method of Jenness (4).

Soluble-membrane protein. This water-soluble protein fraction was isolated from the fat-globule membrane by the method of Herald and Brunner (3), Figure 5. Fresh cream was diluted with three volumes of water and passed through a laboratory-size DeLaval No. 9 separator. This operation was repeated five times. The washed cream was chilled and churned. Free fat was removed by centrifugal separation at 45° C. The membrane was salted out of the buttermilk with $(NH_4)_2SO_4$ (0.5 saturated) and treated with cold ($< 5°$ C.) 35% ethanol in ethyl ether mixture to liberate the lipids of the complex, which were removed in subsequent washings with ethyl ether. A water slurry of the protein was centrifuged at 25,000 × G to separate the soluble protein fraction into the supernatant, which was collected, concentrated, and lyophilized.

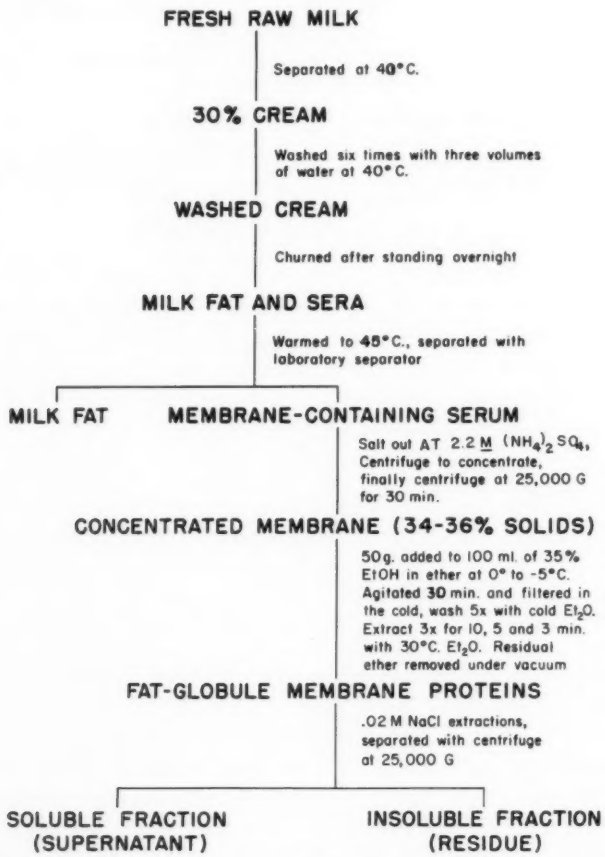
FRESH RAW MILK
Separated at 40°C.
30% CREAM
Washed six times with three volumes of water at 40° C.
WASHED CREAM
Churned after standing overnight
MILK FAT AND SERA
Warmed to 45°C., separated with laboratory separator
MILK FAT
MEMBRANE-CONTAINING SERUM
Salt out AT 2.2 M $(NH_4)_2SO_4$, Centrifuge to concentrate, finally centrifuge at 25,000 G for 30 min.
CONCENTRATED MEMBRANE (34-36% SOLIDS)
50g. added to 100 ml. of 35% EtOH in ether at 0° to -5°C. Agitated 30 min. and filtered in the cold, wash 5x with cold Et_2O. Extract 3x for 10, 5 and 3 min. with 30°C. Et_2O. Residual ether removed under vacuum
FAT-GLOBULE MEMBRANE PROTEINS
.02 M NaCl extractions, separated with centrifuge at 25,000 G
SOLUBLE FRACTION (SUPERNATANT)
INSOLUBLE FRACTION (RESIDUE)

FIG. 5. Schematic diagram showing the isolation of the soluble membrane protein according to Herald and Brunner (3).

ANALYTICAL METHODS

Electrophoretic analyses of the protein fractions were performed with a Perkin-Elmer Model 38-A electrophoresis apparatus at approximately 1° C. in both veronal and HCl-NaCl buffers at pH 8.6 and 2.4 at 22° C., respectively. Ionic strength was adjusted to 0.1. Mobilities were measured from the initial boundary positions on the electrophoretic patterns.

Sedimentation-velocity studies were conducted with a Spinco Model E ultracentrifuge, employing analytical accessories at accelerated rotor speeds (52,640–59,780 r.p.m.) at 20° C. The protein fractions were carried in veronal buffer at concentrations approximating 1.5%. Sedimentation values were reported as S_{20}, uncorrected.

Nitrogen, phosphorus, and fat were determined by conventional procedures. Hexose was determined through its reaction with orcinol, galactose serving as a carbohydrate standard.

TABLE 1

Composition and characteristics of the minor protein fractions of bovine milk

Fraction	Component				Percentage of fraction soluble in 12% TCA solution	
	Nitrogen	Phosphorus	Hexose	Lipid	Original	Rennin-treated
Proteose-peptone	13.70–13.90	1.10	2.90–4.30	1.30	19.35	32.25
σ-proteose	13.72–13.96	0.88	2.8	0	3.98	4.97
Milk component 5 concentrate	12.60	1.50	0.92	0	6.67	7.26
Milk component 5 concentrate (heated)	.....	.....	.....	.....	11.30	14.78
Minor-protein fraction	10.25–10.40	0.63	3.46–6.94	0	41.24	46.67
Soluble membrane-protein	10.35	0.68	2.69–4.04	11.40	6.03	6.00

RESULTS AND DISCUSSION

The composition of the minor-protein fractions, Table 1, shows general similarities. The low nitrogen content and the presence of carbohydrate support their classification as glyco-proteins. A previous report from this laboratory showed that neuraminic acid was a constituent of these interesting protein fractions (8). The fractions contain relatively high concentrations of phosphorus and are correspondingly high in ash. The lipid, found in the proteose-peptone and soluble membrane-protein fractions, represents residual lipids not removed in the preparative procedure. The lipid is tenaciously complexed in the fat-globule membrane and is extremely difficult to remove in its entirety during the isolation of the soluble membrane-protein. An adjustment of the compositional data for this fraction, to correct for the 11.4% of lipid, would bring the nitrogen, phosphorus, and hexose values more in line with those reported for the proteose-peptone fraction.

All of the protein fractions contained 12% TCA-soluble protein, ranging from 4% for σ-proteose to 41% for Weinstein's minor-protein fraction. Fol-

lowing treatment with crystalline rennin, the skimmilk fractions showed increases in the TCA-soluble protein. This was especially significant in the case of fractions receiving heat-treatment (95° C.-30 min.) at some stage in their isolation. The influence of heat-treatment on the TCA-solubility characteristics and on the capacity for rennin to act on the protein is demonstrated by the data for whey component 5 (Table 1), a fraction isolated by a procedure not incorporating the heat-treatment step normally employed in the isolation of the other skimmilk-derived fractions. Approximately 6.6% of the fraction as originally prepared was TCA-soluble. Following the addition of renin to a water solution (pH 7.0) of the protein, 7.2% was TCA-soluble, an increase of ~ 9%. Interestingly, the heated solutions (95° C.-30 min.) contained 11.3% TCA-soluble proteins, and, following the addition of rennin to the cooled solution, the TCA-soluble material increased to 14.8%, an increase of ~ 31%. This observation and the data reported by Jenness (4), showing that heated milk component 5 concentrate would not depress loaf-volume when added to

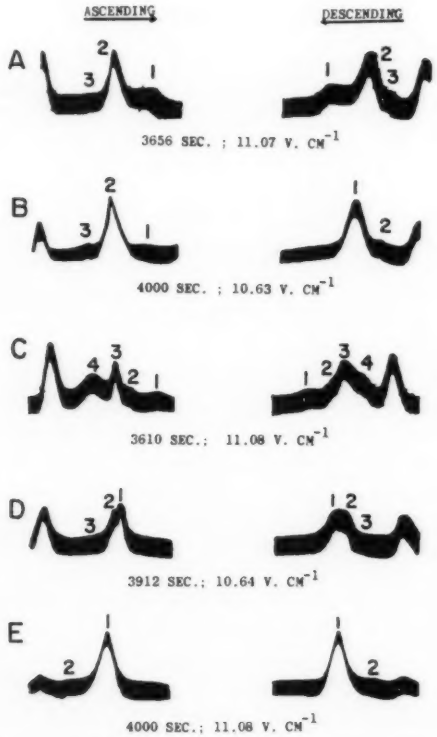

FIG. 6. Electrophoretic patterns of (A) proteose-peptone, (B) σ-proteose, (C) milk component 5, (D) minor-protein fraction, and (E) soluble membrane-protein run in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$; protein conc., 1.5%.

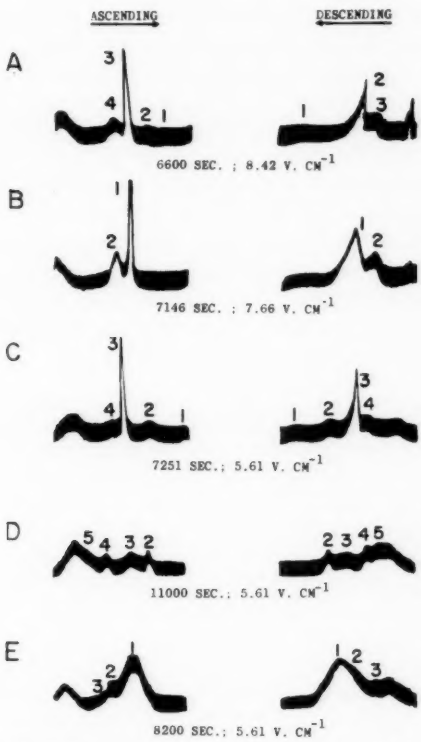

FIG. 7. Electrophoretic patterns of (A) proteose-peptone, (B) sigma proteose, (C) milk component 5 concentrate, (D) minor-protein fraction, and (E) soluble membrane-protein run in HCl-NaCl buffer, pH 2.4, $\Gamma/2 = 0.1$; protein conc., 1.5%.

bread flour, whereas the unheated fraction did so, suggest that the fraction or a component thereof is heat-altered. Rennin did not act on the membrane fraction before or following a heat-treatment.

The high concentration of TCA-soluble protein found in the Weinstein fraction reflects the presence of scission products resulting from the action of rennet on casein (2, 6). These materials constitute a large portion of the Weinstein fraction and, if prepared from rennet whey, the σ-proteose fraction.

The percentage of protein soluble in the 12% A solutions was found to be independent of the total protein concentration at levels ranging from 0.5 to 2% total protein.

Figures 6 and 7 represent free-boundary electrophoretic patterns of the minor-protein fractions in veronal, pH 8.6 and NaCl-HCl, pH 2.4 buffers, respectively. Electrophoretic mobilities and relative areas for the resolved peaks appearing in both the ascending and descending legs in these patterns are tabulated in Table 2. Although differences exist in the electrophoretic charac-

teristics of these fractions, some similarities prevail which seem to be common to all the patterns. Especially is this true for the characteristics of the major electrophoretic peak. Any further attempt to associate a specific peak in one pattern with a peak appearing in another would be hazardous, in view of the electrophoretic complexity of the fractions. Consequently, the patterns are presented primarily for the purpose of demonstrating the over-all electrophoretic differences and/or similarities of the various fractions in two buffers, differing widely in composition and pH.

The temptation to engage in some speculative interpretation of these data can not be denied. The proteose-peptone fraction, representing the total protein residue of heated skimmilk from which casein was removed by I.E.P. precipitation, shows three or four peak areas. The patterns for the σ-proteose fraction,

TABLE 2

Electrophoretic mobility and relative percentage area of the migrating boundaries of the minor protein fractions as determined from the descending-channel electrophoretic mobility patterns shown in Figures 5 and 6

Protein fraction	Buffer-system ($\Gamma/2 = 0.1$)	Migrating boundary [a]							
		1		2		3		4	
		μ [b]	A %	μ	A %	μ	A %	μ	A %
From Figure 5	Veronal, pH [c] 8.6								
Proteose-peptone		(−)7.4 [d]	17.2	4.5	79.3	2.7	3.5		
Row A		6.3 [e]	28.0	3.5	66.7	2.0	5.3		
σ-Proteose		6.6	3.0	4.5	87.8	2.6	9.2		
Row B		5.8	7.0	3.9	85.5	2.0	7.4		
Milk component 5 concentrate		8.8	4.2	6.5	6.1	5.4	39.0	3.6	50.8
Row C		7.2	9.8	5.4	15.9	4.4	52.5	2.8	21.8
Minor-protein fraction		5.4	56.4	4.8	38.7	3.2	4.6		
Row D		5.3	65.6	4.5	30.4	3.1	4.0		
Soluble membrane-protein		4.8	97.4	2.2	2.6				
Row E		4.7	96.5	2.3	3.5				
From Figure 6	HCl-NaCl, pM 2.4								
Proteose-peptone		(+)5.6	2.3	4.5	4.6	3.4	58.5	2.5	34.6
Row A		5.6	5.4	....	....	2.3	70.0	1.5	26.6
σ-Proteose		4.0	56.4	3.1	74.4				
Row B		3.0	43.6	1.7	25.6				
Milk component 5 concentrate		8.7	8.8	6.4	12.9	4.3	68.3	3.4	9.9
Row C		8.4	6.2	5.6	12.8	3.4	65.0	2.5	16.0
Minor-protein fraction		7.6	10.7	4.1	17.0	3.0	24.6	1.8	16.3
Row D		6.4	15.2	3.4	16.6	2.5	18.0	1.4	22.9
Soluble membrane-protein		4.5	78.1	2.8	15.6	1.9	6.3		
Row E		4.3	76.4	3.4	14.6	1.4	9.0		

[a] Designation of boundaries shown in Figures 5 and 6.

[b] Mobility (μ) = cm.^{2}, sec.$^{-1}$, v.$^{-1} \times 10^{-5}$; migrating distances measured from initial boundary.

[c] pH values measured at 22° C.

[d] First row of figures from ascending channel.

[e] Second row of figures from descending channel.

representing the heat-stable protein, salted out of heated, acid whey with $(NH_4)_2SO_4$, showed less area under the leading peak than observed in the patterns for the proteose-peptone. A similar observation was made for the Weinstein minor-protein fraction run in veronal buffer. These observations demonstrate that a fast-moving component, possibly of high phosphorus content with a mobility corresponding to whey component 8, was not quantitatively salted out of the sera by ammonium sulfate. The closely associated pair of peaks in the Weinstein fraction (veronal) represent the major proteose-peptone component and scission products from the action of rennet on casein, principally the macroglycopeptide. The heterogeneity of this fraction was most obvious in patterns obtained in the HCl-NaCl buffer, pH 2.4. The apparent reduction in the relative refractive area can be accounted for on the basis that the macroglycopeptide does not migrate from the initial boundary area under these conditions (2).

The Jenness fraction shows at least four electrophoretic peaks. Obviously, milk component 5 is not the only protein in the concentrate. It appeared to be contaminated with a small quantity of β-lactoglobulin, which appears in the electrophoretic patterns and in the sedimentation diagram in a position normally assigned to this protein. Interestingly, the eluate (pH 2.5) from the IRC-50 column, employed in the preparation of this fraction, contained a floc which was not apparent in the protein solution prior to its passage over the column. Nor was it possible to produce the floc by adjusting the solution to pH 2.5. Could it be that one of the components retained on the column serves as a stabilizing agent for the entire system? Experiments are in progress in this laboratory to elucidate the characteristics of the interesting protein system constituting the Jenness fraction.

Compositional and electrophoretic characteristics of the soluble membrane-protein were approximately similar to those for the fractions obtained from skimmilk.

TABLE 3

Sedimentation constants for the minor protein fractions of bovine milk measured from the sedimentation-velocity patterns shown in Figure 3

Fraction	Sedimentation boundary	
	Peak 1	Peak 2[a]
	$(S_{20})^b$	
Proteose-peptone Row A	2.64	0.77
Sigma proteose Row B	2.86	0.83
Milk component 5 concentrate Row C	5.88	0.88
Minor-protein fraction Row D	2.83	0.84
Soluble membrane-protein Row E	17.60	9.05

[a] Major component.

[b] $S_{20} = S_{20} \times 10^{-13}$; run in veronal buffer at pH 8.6, $\Gamma/2 = 0.1$; 1.5% protein conc.; uncorrected for buffer viscosity.

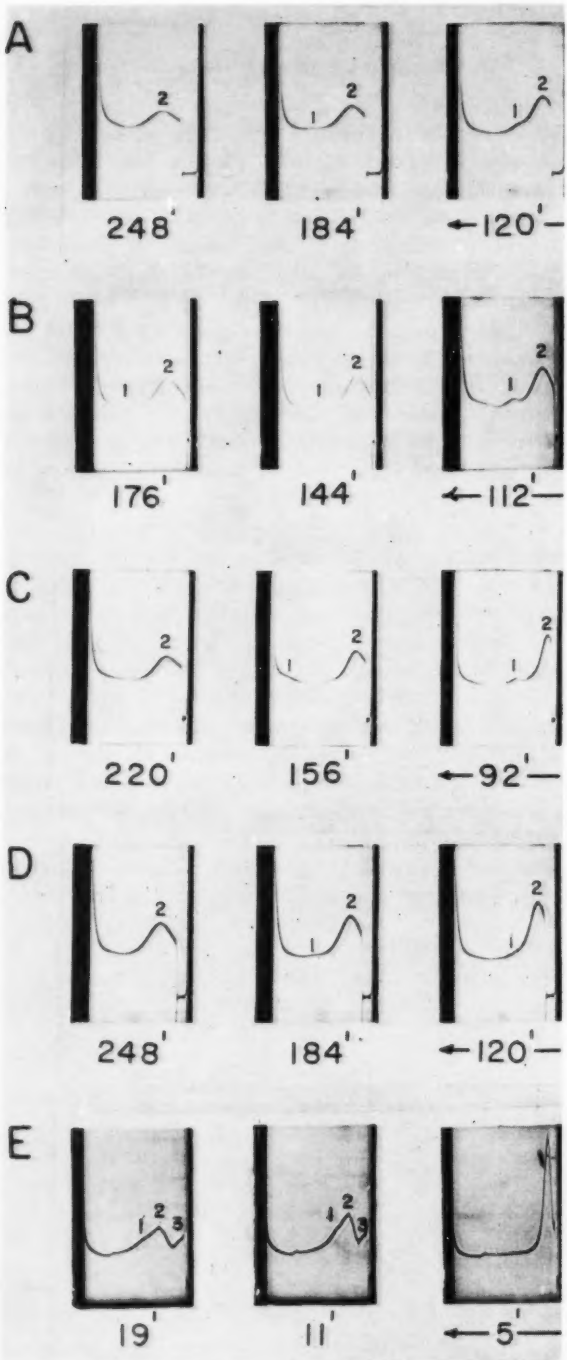

FIG. 8. Sedimentation-velocity diagrams of (A) proteose-peptone (B) sigma proteose, (C) milk component 5 concentrate, (D) minor-protein fraction, and (E) soluble membrane-protein run in varonal buffer at pH 8.6, $\Gamma/2 = 0.1$. Protein conc. was 1.5% for A, B, C, and D and 1.0% for E. Rotor speed for A, B, C, and D was 59,780 r.p.m. and 52,640 r.p.m. for E. Angle of the phase plate was 50° for A, B, C, and D, and 45° for E. Small, unlabeled peak appearing in the diagram in Row E represents an optical flow.

Figure 8 and Table 3 show sedimentation-velocity diagrams and constants (S_{20}, uncorrected), respectively, for the minor-protein fractions. The sedimentation diagrams of the skimmilk-derived fractions exhibited a common major sedimentation boundary of about .8 Svedburg's and, in the heat-treated fractions, a slower-moving, minor boundary of about 2.8 Svedburg's. These values agree well with those reported by Aschaffenburg for the σ-proteose and by Jenness for component 5 concentrate. The diagrams for the membrane protein reflect molecular species differences between this fraction and those previously mentioned and show sedimentation coefficients in the order of 10-20 S_{20} units.

Milk component 5 concentrate and the soluble membrane-protein fractions were isolated by methods not employing the use of high temperatures. For the purpose of making a more comparable evaluation of these fractions with those in which exposure to high temperatures constituted a step in the isolation procedure, water solutions of these two preparations were heated to 95° C. for

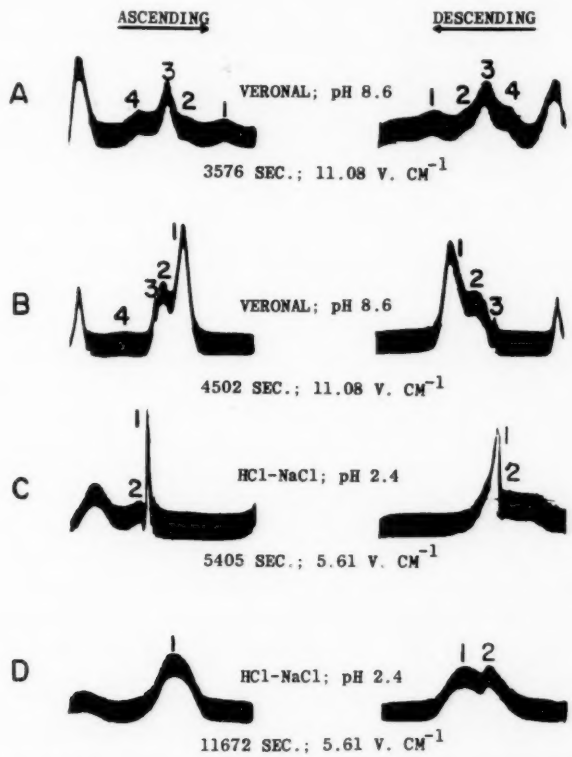

FIG. 9. Electrophoretic patterns of whey component 5 and the soluble membrane-protein following heating at 95° C. for 30 min.: A and C—milk component 5 concentrate, B and D—soluble membrane-protein; conc., 1.5%.

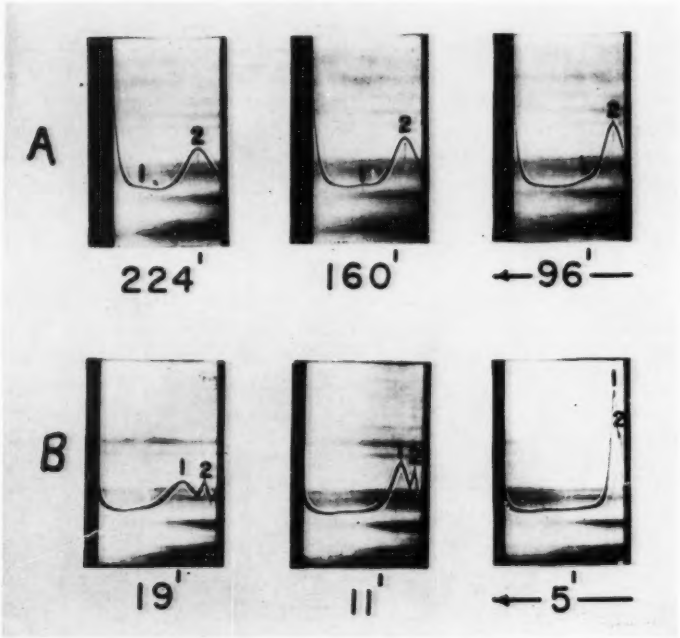

FIG. 10. Sedimentation-velocity diagrams of (A) milk component 5 concentrate and (B) soluble membrane-protein following heating at 95° C. for 30 min. Protein conc., 1.5%; angle of the phase plate, (A) 60° and (B) 50°; temp. 20° C.; speed, (A) 59,780 and (B) 52,640 r.p.m.; time interval as indicated; veronal buffer at pH 8.6, $\Gamma/2 = 0.1$. Small, unlabeled peak appearing in the diagram in Row B represents an optical flow.

30 min., dialyzed against buffer, and re-examined electrophoretically and ultracentrifugally (Figures 9 and 10, respectively). Heating of the proteins resulted in a general decrease in the electrophoretic mobilities of the constituent components. Specifically, the Jenness fraction showed a reduction in the area of Peaks 1 and 4, whereas the membrane protein showed an increased resolution in veronal buffer and a corresponding decrease in acid buffer. The sedimenting boundary ascribed to β-lactoglobulin was absent from the sedimentation diagram of the Jenness fraction. But a new boundary, similar to the fast-moving boundary observed in the heat-isolated fractions ($S_{20} = 2.8$), was observed. The sedimenting boundaries of the membrane fraction were more discernible and somewhat slower, possibly an indication of the dissociating influence of heat on this fraction.

CONCLUSIONS

The four minor-protein fractions isolated from skimmilk were heterogenous systems, reflecting the procedures employed in their isolation. A major electrophoretic and ultracentrifugal peak appeared to be a common component in these fractions.

The soluble membrane-protein fraction, although possessing compositional and electrophoretic characteristics similar to the skimmilk-derived fractions, showed marked differences in sedimentation characteristics.

The application of heat (95° C.-30 min.) during or subsequent to the isolation of these fractions produced alterations in the properties of the protein constituents, as demonstrated by minor changes in their electrophoretic and ultracentrifugal characteristics and, with the exception of the membrane fraction, their susceptibility to the action of rennin.

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HEAT STABILITY OF CASEIN IN THE PRESENCE OF CALCIUM AND OTHER SALTS [1]

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SUMMARY

The extent of calcium binding and the heat stability of colloidal suspensions of freeze-dried casein in calcium hydroxide solution, with added phosphates, citrates, and chlorides in some tests, was studied. The calcium-binding power of freshly prepared freeze-dried casein was greater (pK 4.5) than that reported for other casein preparations. However, the pK value decreased during storage of the casein at room temperature and within 1 or 2 mo. attained a value ($pK = 3.0 \pm 0.3$) in agreement with published values.

The heat stability of simple casein caseinate suspensions was inversely related to the extent to which the casein was saturated with calcium, and this relation appeared to be linear at calcium levels sufficient to give over 80% saturation. The heat stability of more complex suspensions was inversely related to the ratio of soluble or ionic calcium to soluble phosphate in the suspension.

Chlorides and phosphates of sodium, potassium, and ammonium displaced calcium from caseinate to about the same extent, but ammonium ion appeared to have a specific depressant effect on heat stability.

In an earlier paper (14), it was suggested that the heat stability of milk varied with a multiplicity of factors which might differ in relative importance between samples. If so, identification of these factors will be greatly facilitated by use of simple model systems containing milk proteins. Colloidal suspensions of acid casein, which do not involve extensive modification or fractionation of the casein itself, probably provide the most suitable system. Studies of the heat stability of such suspensions were, therefore, undertaken.

The binding of calcium by casein in calcium hydroxide-casein mixtures has been studied extensively (2, 3, 7, 19, 20), but data on the heat stability of calcium casein solutions are quite limited (6, 15, 17, 18). The present paper reports on the interrelations between calcium binding and heat stability in colloidal suspensions of casein in calcium hydroxide, with and without added chlorides, phosphates, and citrates.

MATERIALS AND METHODS

Acid casein was precipitated from skimmilk by the addition of hydrochloric acid to pH 4.6, washed several times with water, frozen, and dried under vacuum. The dried product was ground with a pestle and stored at room temperature. Several batches of freeze-dried casein were used in this work; differences between batches were less than the experimental error plus the effect of aging (vida infra). Except where otherwise stated, casein preparations were from 1 to 4 mo. old.

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For dispersion of the casein, measured amounts of saturated calcium hydroxide solution, filtered through two thicknesses of Whatman No. 12 paper (and stored under oil if not used immediately) were mixed with a weighed amount of casein and the mixture shaken for 15 to 18 hr. Aliquots of this colloidal suspension were adjusted to the desired pH with 1 *N* hydrochloric acid or sodium hydroxide, and to the desired chloride, phosphate, and citrate content, and diluted to contain 2.8% casein (w/v, corrected for 6% moisture).

Calcium was determined by a turbidimetric method (9); total calcium by analysis of an acidified aliquot of the freshly filtered calcium hydroxide solution, soluble calcium by analysis of ultrafiltrates prepared as previously described (16). Ionic calcium was determined with murexide (16), by comparison with standards of the same ionic strength. Ionic strength varied between 0.008 and 0.05, except in a few tests at high chloride concentrations. Total (trichloracetic acid soluble) and soluble (ultrafiltrate) inorganic phosphate were determined colorimetrically (10). Heat stability was measured as the time at 140° C. required to develop visible particles in milk flowing down the wall of the test-tube (16).

Bound calcium was calculated as the difference between total and ultrafiltrate calcium. However, this calculation was not permissible when total and soluble inorganic phosphate values differed, indicating that calcium phosphate had precipitated. Although Zittle *et al.* (20) observed binding of phosphate itself, in the present tests differences between total and ultrafilterable phosphate were never observed in mixtures in which the estimated $[Ca^{++}]^3[PO_4^{=}]^2$ and $[Ca^{++}][HPO_4^{=}]$ products were below those expected to induce precipitation of a calcium phosphate.

RESULTS

Binding of calcium. In the absence of other salts, the extent to which calcium was bound to casein was a function of the concentration of calcium present and the pH. At fixed pH, the reciprocal of the moles of calcium bound per 10^5 g. casein (r) at room temperature was a linear function of the reciprocal of moles of free calcium present (A) (*cf.* 8). Extrapolation to the axes gave a dissociation constant for a fresh batch of freeze-dried casein of $pK = 4.36 \pm 0.10$ at pH 6.5, and of $pK = 4.52 \pm .12$ at pH 6.95. Caseins that had been stored at room temperature for 1 to 10 mo. had pK values from 2.66 to 3.80 at pH 7. The variability in these tests, inclusive of variation between batches of casein, was higher than suggested by the standard deviations quoted above, but nevertheless the data indicated that the pK for calcium caseinate decreased by 0.6 unit or more during the first month of storage at room temperature, with a further slow decline to $pK = ca\ 3.0 \pm 0.3$. Published values (3, 19) vary from 2.39 to 3.34.

The maximum amount of calcium bound by 10^5 g. of fresh casein was $38.5 \pm 0.5M$ at pH 6.95, and 33.1 ± 0.4 at pH 6.5. Older caseins (1 to 10 mo.) bound from 33 to 36 $M/10^5$ g. at pH 7, with no significant trend with time of storage. The effect of pH on the maximum amount of calcium bound by casein was similar to that reported by Zittle *et al.* (20).

If the calcium concentration was less than that required to saturate the casein at room temperature, more calcium was bound at 93° C. than at room temperature (Table 1). The increase was small under all conditions tested, but was sufficient to cause the 1/r vs. 1/A relation to deviate sharply from linearity (Figure 1).

TABLE 1

Effect of temperature on the amount of calcium bound by casein
(Averages of 3 tests, pH 6.5)

Calcium added *(mM/liter)*	Calcium in ultrafiltrate *(mM/liter)*		Calcium bound *($M/10^5$ g.)*	
	25° C.	93° C.	25° C.	93° C.
7.5	0.5	0.1	25.1	26.2
7.8	0.6	0.2	25.8	27.2
9.2	1.1	0.8	28.6	29.6
12.2	3.1	3.1	32.2	32.4

Addition of chlorides or phosphates of sodium, potassium, or ammonium to colloidal casein–calcium hydroxide suspensions in which the casein was not saturated with calcium, displaced calcium from the casein (Figure 2). Added citrates sequestered calcium and thus greatly reduced the amount bound by casein (Figure 2). These effects appeared to be independent of the cation present.

Heat stability. At pH 6.5, 7.0, or 7.5, and calcium levels above 7.5 m*M*/liter of colloidal casein–calcium hydroxide suspension, the heat stability was inversely related to the extent to which casein was saturated with calcium (Table 2).

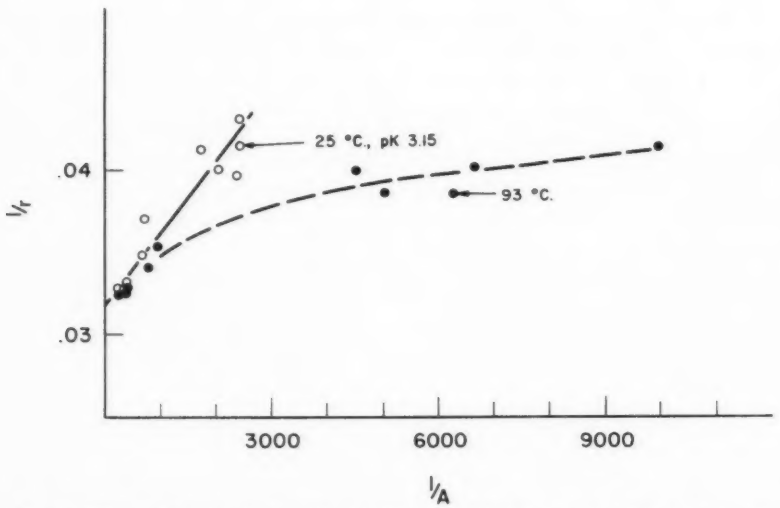

FIG. 1. Effect of temperature on the binding of calcium by casein. Data are from same tests as in Table 1, but not averaged: pH 6.5; ○—25° C., ●—93° C.

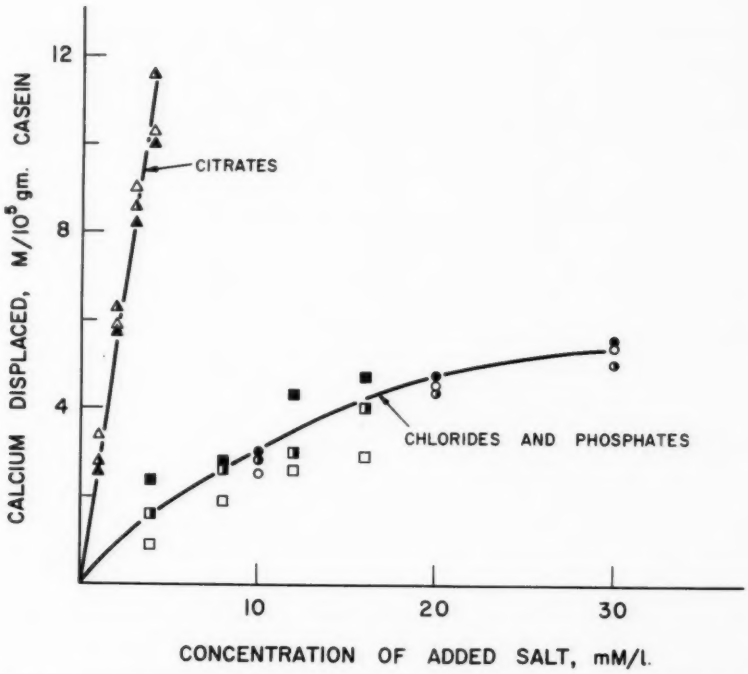

FIG. 2. Displacement of calcium from casein by sodium, potassium, and ammonium salts (i.e., bound calcium of control minus bound calcium of sample with added salt). Calcium concentration 7 to 8 m*M*/liter, pH 6.5, temperature 25° C.

○, ◑, ●—Na, K, and NH_4 chlorides, respectively
□, ◧, ■—Na, K, and NH_4 phosphates, respectively
△, ◭, ▲—Na, K, and NH_4 citrates, respectively

At fixed calcium level, heat stability decreased with decreasing pH, becoming essentially zero at pH 6.0 (Table 2). The heat stability of colloidal casein–calcium hydroxide suspensions also decreased with increasing age of the casein preparation; e.g., casein 80% saturated with calcium had a heal stability of 12 min. when 2 to 6 wk. old, and of only 8 min. when 20 to 24 wk. old (average of three tests in each period). With very fresh casein a heat stability of 20 min. at 80% saturation was observed (single test).

When other salts were added to the colloidal suspension of casein in calcium hydroxide, the heat stability was influenced both by the anion and by the cation (Figure 3). Sodium and potassium phosphates greatly increased the heat stability; ammonium phosphate did not. The heat stability of suspensions containing ammonium citrate was also lower than that of similar suspensions containing sodium or potassium citrate (Figure 3). Addition of 10 m*M*/liter sodium or potassium chloride did not significantly affect the heat stability of casein–calcium hydroxide suspensions; higher levels (20 and 30 m*M*/liter) de-

TABLE 2

Effect of saturation of casein with calcium, and of pH, on heat stability of colloidal suspensions of casein in calcium hydroxide

pH	Total calcium (*mM/liter*)	Bound calcium ($M/10^5$ g.)	Saturation [a] (%)	Heat stability (*min.*)
7.5	7.8	26.3	70.9	26
	9.8	30.5	82.3	16.5
	12.6	36.0	97.1	1
	16.0	36.9	99.4	0.5
	19.5	36.4	98.0	nil
7.0	7.8	26.0	76.6	21
	9.8	30.5	90.0	2
	12.6	33.7	99.4	0.5
	16.0	33.7	99.4	0.25
	19.5	32.9	96.9	nil
6.5	7.8	24.4	85.2	7.5
	9.8	27.3	95.6	0.75
	12.6	27.6	96.3	nil
	16.0	27.6	96.3	nil
	19.5	28.1	98.1	nil
6.0	7.8	19.1	85.7	0.5
	9.8	18.6	83.3	0.25
	12.6	20.1	90.5	nil
	16.0	22.3	100	nil
	19.5	22.3	100	nil

[a] Per cent of maximum as determined by extrapolation to infinite calcium concentration.

creased it by about 25%. Ammonium chloride decreased heat stability approximately 30, 60, and 80% when present at 10, 20, and 30 m*M*/liter concentrations (single test).

Since the heat stability of colloidal suspensions containing only casein and calcium hydroxide was related to the per cent saturation of casein with calcium (Table 2), and calcium binding, and hence per cent saturation of casein with calcium was related to the reciprocal of the free calcium present at room temperature (*cf.* 8), heat stability of these simple mixtures was also related to $1/Ca_{sol}$. However, this relation was not apparent when phosphate or citrate ($1/Ca^{++}$ substituted for $1/Ca_{sol}$ in the presence of citrate) was also present.

The heat stability of colloidal suspensions containing casein, calcium, and phosphate was inversely and linearly related to the Ca/P ratio in the ultrafiltrate. Data for 29 tests, including some in which calcium phosphate precipitated, gave a correlation between heat stability and the Ca_{sol}/P_{sol} ratio of −0.96 (Figure 4). However, the per cent saturation of casein with calcium was also related to the Ca_{sol}/P_{sol} ratio. These two factors could not be separated experimentally, but their individual effects can be approximated by standard statistical methods (partial correlation analysis). Among the 29 tests, there were 14 in which no calcium phosphate precipitated (hence, bound calcium could be estimated), and statistical analysis of the data from these yielded the following results:

Crude (apparent) correlation between Ca_{sol}/P_{sol} and heat stability	−0.97

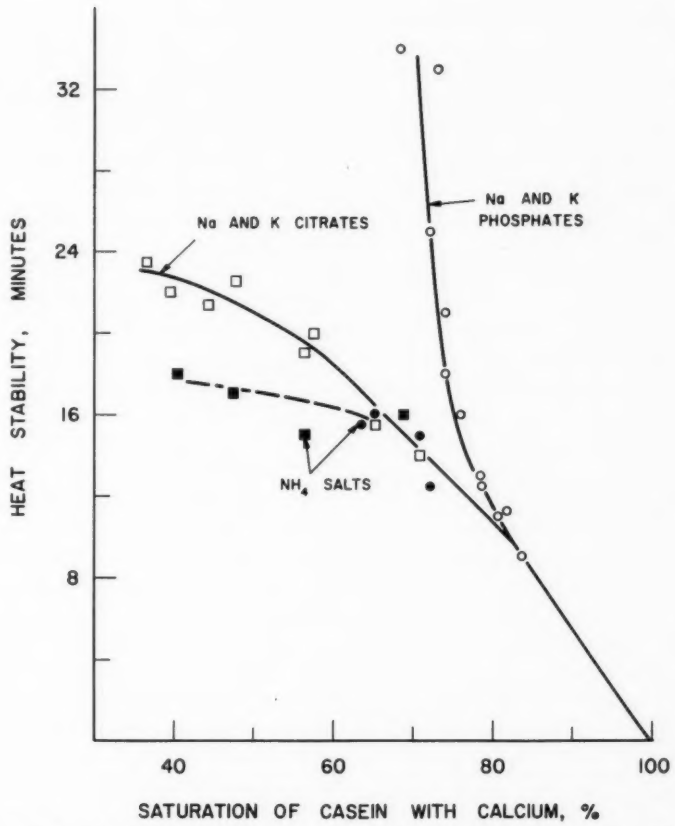

FIG. 3. Effect of added citrate (0 to 4 m*M*/liter) and phosphate (0 to 16 m*M*/liter) on the heat stability of colloidal suspensions of casein. Calcium concentration 7 to 8 m*M*/liter, pH 6.5, temperature 25° C.

○—Na or K phosphate ●—NH_4 phosphate
□—Na or K citrate ■—NH_4 citrate

The linear relation above 80% saturation (no points shown) was established by several tests with casein–calcium hydroxide suspensions without added salts.

The corresponding crude regression coefficient of heat stability on Ca_{sol}/P_{sol}	-70.0 ± 7.0
The same correlation corrected for the effect of per cent saturation on Ca_{sol}/P_{sol}	-0.83
The corresponding corrected regression coefficient of heat stability on Ca_{sol}/P_{sol}	-48.7 ± 9.9

It is thus apparent that the correlation and regression are inflated by the underlying relation between per cent saturation and Ca/P, and the screening out of this relation gives lower and more realistic values.

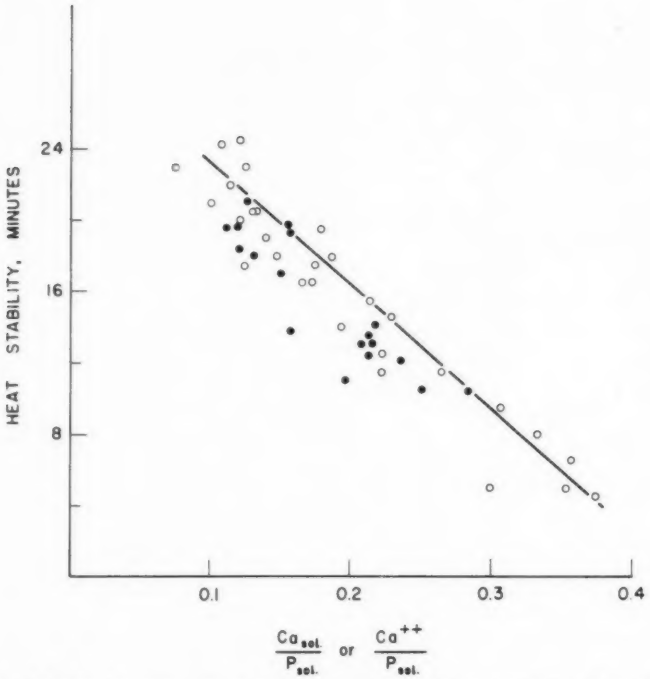

FIG. 4. Relation between the heat stability of colloidal suspensions of casein and the ratio of soluble (or ionic) calcium to soluble phosphate. Calcium concentration varied from 7 to 18 m*M*/liter, pH 6.5.

○—casein-calcium-phosphate suspensions

●—casein-calcium-phosphate-citrate suspensions

The heat stability of colloidal suspensions containing citrate in addition to casein, calcium, and phosphate was also inversely related to the Ca^{++}/P_{sol} ratio (Figure 4). There was no apparent relation between the heat stability of these suspensions and the $Ca_{sol}/P_{sol} + Citrate_{sol}$ ratio.

DISCUSSION

Freshly prepared freeze-dried casein had a greater affinity for calcium (pK = 4.5) than had the commercial (3) or solvent-dried (19) caseins tested by other authors. However, freeze-dried caseins stored 1 to 10 mo. at room temperature had pK values (*ca* 3.0 ± 0.3, range 2.7 to 3.8) that did not differ significantly from previously reported values (3, 19). The high value for freshly prepared freeze-dried casein suggests that native casein has a greater affinity for calcium than indicated by published data on acid casein.

The maximum amount of calcium bound by freeze-dried casein varied from 33 to 36 $M/10^5$ g. casein at pH 7.0 (38 for freshly prepared casein). Assuming

a molecular weight of 33,000, the calcium bound is 11 to 12 *M/M* casein. This value is lower than that obtained by Chanutin *et al.* (3) [*cf.* also (7)], but is in reasonable agreement with a value of 10 *M/M* estimated from the data of Zittle *et al.* [(20) Figure 4].

Heat stability of the colloidal suspensions of casein in calcium hydroxide appeared to be largely dependent upon (a) the per cent to which the casein was saturated with calcium, and (b) the ratio of soluble calcium to soluble phosphate in the suspensions. The amount of calcium bound to casein in milk has been estimated by assuming a constant Ca/P ratio in the colloidal calcium phosphate (4, 5, 13) but, in view of the variability in composition of calcium phosphate precipitates (1), these assumptions are difficult to justify. Pyne and McGann (11) have recently introduced an acidification-dialysis technique for preparing milk containing no colloidal calcium phosphate, but this method has not been applied to a study of the casein-bound calcium in a range of milks. At present, therefore, it is not possible to decide whether the degree of saturation of casein with calcium in normal milks can be a factor in determining their heat stability. On the other hand, the relation between the Ca_{sol}/P_{sol} ratio and the maximum heat stability of normal milk (14) was similar to that observed in the present tests. Colloidal calcium phosphate is thought to be a major factor controlling the heat stability of normal milks (12), but precipitation of calcium phosphate in the colloidal suspensions of calcium caseinate did not alter the relation between the Ca_{sol}/P_{sol} ratio and the heat stability.

Added salts probably affect the heat stability of colloidal suspensions of casein by displacing calcium from the casein and, when phosphate is present, by affecting the soluble (or ionic) calcium/soluble phosphate ratio. However, ammonium ion displaced calcium from casein to the same extent as did sodium or potassium, but did not increase heat stability correspondingly. Ammonium ion probably had a specific depressant effect on the heat stability of these colloidal suspensions.

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ACID PHOSPHATASE IN CREAM AND IN SKIMMILK

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SUMMARY

Acid phosphatase activity in cream is about twice that in skimmilk. The phosphatase in both is relatively heat-stable; 70% of the activity remained after heating at 65° C. for 15 min. The enzymes from raw cream and raw skimmilk were each purified threefold. The cream enzyme was concentrated in the insoluble residue obtained by centrifuging the buttermilk fraction of washed cream at 50,000 × G for 1 hr. When skimmilk was centrifuged under the same conditions, most of the enzyme activity remained in the supernatant solution, which was further purified by ammonium sulfate treatment to yield a soluble form of acid phosphatase. Ultracentrifuge fractionation of buttermilk failed to concentrate the cream enzyme activity in a single fraction. Acid phosphatase, alkaline phosphatase, and xanthine oxidase activities were broadly distributed in these fractions. pH activity curves, K_M values, and the effect of inhibitors are reported for the purified acid phosphatase of cream and milk. These studies showed no significant difference between the two enzyme preparations.

The occurrence of acid phosphatase in whole cow's milk has been reported by Mullen (9), who investigated some of the properties of the enzyme but did not attempt its purification. The distribution of acid phosphatase in cream and in skimmilk had not been studied. Such data would be of interest for comparison with xanthine oxidase and alkaline phosphatase distribution figures, because related studies (7, 8, 13) indicate that many of the enzymes of milk occur in the fat globule membrane of cream. The present report describes acid phosphatase in cream and skimmilk, and compares some properties of the enzymes from the two sources.

METHODS

Assay of alkaline phosphatase. Enzyme activity was determined by a modification of the method used by Zittle and DellaMonica (11). The test material was incubated 5 min. at 38° C. with 0.00083 *M* disodium phenyl phosphate, 0.0025 *M* $MgCl_2$, and 0.067 *M* ethanolamine-HCl buffer, pH 9.8. The enzyme preparation made up 1 ml. of this 6-ml. incubation mixture. The liberated phenol was estimated colorimetrically. Activities are reported as micromoles phenol released when 1 ml. enzyme preparation is incubated with 5 ml. substrate for 1 hr. at 38° C. The unit of activity is defined as the amount of enzyme that will hydrolyze 1 μM of substrate under the same conditions.

Assay of acid phosphatase. Two methods were used. In the first part of the experimental work the enzyme was determined by incubating the sample for 1 hr. at 38° C. with 0.00417 *M* disodium phenyl phosphate and 0.0417 *M* succinic acid-borax buffer, pH 4.0 (5). With cream and all fractions of cream and skim-

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milk, there was a linear relationship between amount of enzyme and phenol released. With skimmilk, there was some departure from linearity with the larger amounts of sample. Conversion of phenol to activity units was the same as for alkaline phosphatase. Subsequently, the acid phosphatase was estimated by Lowry's method (2), with disodium *p*-nitrophenyl phosphate as the substrate. This method was more rapid than the previous one, and at pH 5.2, the optimum pH with this substrate, precipitates of casein when present were finer and more easily suspended. One-tenth-milliliter enzyme was added to 1 ml. buffer-substrate containing 0.005 *M* disodium *p*-nitrophenyl phosphate and 0.1 *M* acetate buffer, pH 5.2. After 30 min. at 38° C., the reaction was stopped by the addition of 3 ml. 0.25 *N* NaOH, bringing the pH to 12.0. Blanks were included in every assay performed. Free nitrophenol was determined, using a Beckman Spectrophotometer, Model DU,[2] with a 1-cm. light path at 410 mμ. Activities are expressed as micromoles nitrophenol released when 0.1 ml. enzyme is incubated with 1 ml. substrate for 30 min. at 38° C. The unity of activity is defined above.

Assay of xanthine oxidase. The method of Zittle *et al.* was used (12).

Assay of protein. Protein was estimated by the method of Lowry *et al.* (6).

Raw skimmilk and raw cream from pooled milk was supplied by a local dairy.

EXPERIMENTAL PROCEDURE AND RESULTS

1. *Distribution.* Acid phosphatase of skimmilk and cream is of relatively low activity. When assayed with disodium phenylphosphate substrate, the activity in cream was 0.68 unit per milliliter; in skimmilk the activity was 0.37 unit per milliliter. Thus, in whole milk with a skimmilk:cream ratio of 7:1, 21% of the activity is located in the cream and 79% in the skimmilk. These values varied considerably with different samples and in some batches approached a 1:1 proportion.

The variable distribution of the acid phosphatase between skimmilk and cream appears from the work of others. In one instance (1) cream was reported to contain the highest activity, in the other instance (3a) the skimmilk.

2. *Heat treatment.* Mullen (9) reported that the acid phosphatase of whole cow's milk retained 80-90% of its activity under pasteurization conditions (62.8° C. for 30 min.), whereas the alkaline phosphatase was completely destroyed. This interesting property has been confirmed. Similar results have been reported by others (1, 3a). When skimmilk is heated at 65° C. for 15 min., 67% of the acid phosphatase activity remains. Cream retains 72% of its acid phosphatase activity; whereas, the purified buttermilk preparation retained only 36% of the activity. The alkaline phosphatase in all of these materials was completely destroyed.

3. *Preparation of acid phosphatase from cream.* Preparation of acid phosphatase from cream involved washing by dispersing 40% raw cream in two parts water and centrifuging for 15 min. at 2,000 G at 4° C. After repeating

[2] It is not implied that the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

the centrifuging step, the washed cream was churned with one part water to give buttermilk. The buttermilk was separated from the milk fat by decantation. Small particles of milk fat were removed by centrifuging for 15 min. at 2,000 G. The buttermilk was then centrifuged at 50,000 G for 1 hr., a procedure which sedimented approximately 90% of the acid phosphatase. Each step of the procedure was studied in detail, to determine the optimum conditions for the purification of the acid phosphatase.

As alkaline phosphatase is present in cream and has been extensively studied (7, 8, 13), its activity was also determined. Some interesting differences between the two enzymes were revealed in the fractionation studies using the ultracentrifuge. When these differences became apparent, a few xanthine oxidase activities were determined to se whether this enzyme would be distributed like acid phosphatase or alkaline phosphatase.

(a) *Washing of cream.* Table 1 shows that 50% of the acid phosphatase and 50% of the alkaline phosphatase is removed by washing. Zittle *et al.* (13) found that 75% of the alkaline phosphatase was removed by washing the cream four times. To avoid such large losses, the cream was washed only twice in these procedures.

TABLE 1

Acid phosphatase and alkaline phosphatase in cream and washed cream

	Acid phosphatase	Alkaline phosphatase
	(units/1 ml.)	*(units/1 ml.)*
Cream	.684	55.4
1st washed cream	.518	40.4
1st wash H_2O	.240	11.4
2nd washed cream	.351	28.8
2nd wash H_2O	.130	8.2

Disodium phenyl phosphate substrate was used. Results are calculated to the volume of the original cream.

An attempt was made to increase the yield of acid and alkaline phosphatase in buttermilk by washing with isotonic and hypertonic solutions that might be expected to retain the microsomal structure of the fat globule membrane (4). Accordingly, 0.25 *M* sucrose, 0.5 *M* sucrose, 0.15 *M* NaCl, and 0.45 *M* NaCl solutions were used. Enzyme losses, however, were identical to the loss when water alone was used to wash the cream.

(b) *Churning of cream.* After churning the washed cream, 103% of the original acid phosphatase and 65% of the alkaline phosphatase were present in the buttermilk (Table 2). The butter was washed once with water; additional activity was also obtained by melting the butter at 38° C. and siphoning off the serum. The buttermilk, wash, and serum account for 131% of the initial acid phosphatase and 74% of the alkaline phosphatase. The high yields of acid phosphatase exceeding those measured initially suggest the presence of an acid phosphatase inhibitor in cream.

(c) *Fractionation of buttermilk in the ultracentrifuge.* The acid phosphatase activity in the buttermilk was studied in the ultracentrifuge and its

TABLE 2

Acid phosphatase and alkaline phosphatase in washed cream and buttermilk fractions

	Total volume	Acid phosphatase			Alkaline phosphatase		
		Units per ml.	Total units	Per cent of total	Units per ml.	Total units	Per cent of total
	(ml.)						
Washed cream [a]	750	.266	200	100	25.4	19,050	100
Buttermilk	430	.480	206	103	28.8	12,400	65
Butter wash	107	.099	10.7	5	5.76	617	3
Butter serum	57	.820	46.7	23	21.2	1,207	6

[a] Washed cream was diluted with one part water. Disodium phenyl phosphate substrates were used for assays.

activity compared with that of alkaline phosphatase and xanthine oxidase. The Spinco Model L Ultracentrifuge [2] (Rotors No. 40 and No. 21) was used for this work. The starting material was buttermilk prepared from cream washed in 0.25 *M* sucrose. This was first centrifuged at 2,000 G for 15 min. to remove unchurned cream and butter and then centrifuged according to the procedure shown in Table 3. The results are given in Table 4. After each centrifugation, the supernatant was decanted and the pellet dissolved in 0.25*M* sucrose. The supernatant was then recentrifuged at a higher centrifugal force. The unit, gravity (G) × min. (3), is used as a measure of centrifugation. Neither acid phosphatase nor alkaline phosphatase was obtained in a single fraction (Figure 1). Their broad distribution suggests that these enzymes are associated with

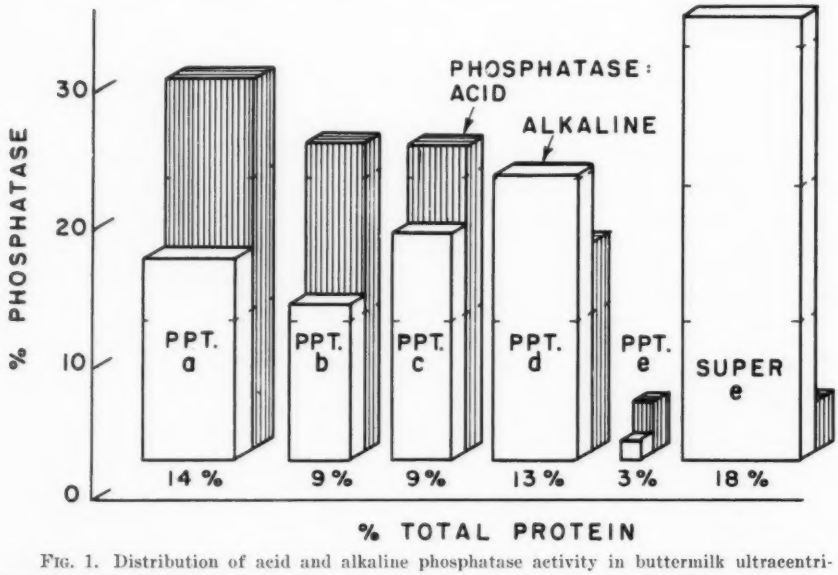

FIG. 1. Distribution of acid and alkaline phosphatase activity in buttermilk ultracentrifuge fractions (Table 3 gives procedure).

TABLE 3

Fractionation procedure for washed cream buttermilk

Fraction	Head No.	Centrifugal force at maximum speed [a]	Duration of centrifugation at maximum speed	Total (G-min. $\times 10^3$)
		($G \times 10^3$)	*(min.)*	
a	21	11.4	8	91.2
b	21	11.4	16	182.4
c	21	26.3	10	263.0
d	40	105.4	15	1,581.0
e	40	105.4	30	3,162.0

[a] Head 40 reaches top speed in 5 min., whereas Head No. 21 takes 20 min.

large micelles having a wide spectrum of size and density. In similar studies, Morton (8) centrifuged washed cream buttermilk and compared the two fractions obtained by centrifuging at 630×10^3 G $\times$ min. and $2,100 \times 10^3$ G $\times$ min. with the final supernatant. The first two fractions had properties that indicated that they were akin to microsomes. The supernatant contained only 8% of the total alkaline phosphatase and was assumed to contain no microsomes.

Table 4 presents the protein, acid phosphatase, alkaline phosphatase, and xanthine oxidase contents of the supernatants and precipitates obtained by the procedure outlined in Table 3. Great differences are evident in the sedimentation patterns of alkaline phosphatase on one hand and xanthine oxidase and acid phosphatase on the other. This is most apparent in supernatant *d* obtained after four successive centrifugations. This supernatant contained 39% of the

TABLE 4

Acid phosphatase, alkaline phosphatase, xanthine oxidase, and protein after centrifugal fractionation of buttermilk

Fraction	Acid phosphatase [a]	Alkaline phosphatase [a]	Xanthine oxidase	Protein
	(units/ml)	*(units/ml)*	*(units/ml)*	*(mg/ml)*
Buttermilk	.273	12.24	40.9	1.39
a supernatant	.174	10.54	29.5	.86
ppt.	.073	1.82		.19
b supernatant	.105	8.74		.62
ppt.	.058	1.47		.123
c supernatant	.042	7.34	4.0	.56
ppt.	.062	2.10		.125
d supernatant	.013	4.79	0.56	.29
ppt.	.042	2.58		.18
e supernatant	.011	4.06		.26
ppt.	.010	0.19		.028

[a] Determined with disodium phenylphosphate substrate. Results are calculated to the volume of the original buttermilk.

original alkaline phosphatase as compared to 4.7% of the acid phosphatase and 1.4% of the xanthine oxidase. The enzymes deposited in the pellet at 1,581 × 10^3 G × min. (Fraction *d*) could be associated with cellular particles such as microsomes. The alkaline phosphatase remaining in solution may be a second enzyme or the same enzyme in a soluble form. By centrifuging washed cream buttermilk at 1,140 × 10^3 G × min., Sasaki (10) presented evidence that the alkaline phosphatase in the supernatant differed from the alkaline phosphatase in the precipitate.

The above experiments pointed out conditions for the separation of acid phosphatase from washed cream buttermilk by which the specific activity of the buttermilk was increased from .018 to .062, a purification of better than three-fold. The procedure could be carried out in water as well as 0.25 *M* sucrose, indicating that the insoluble acid phosphatase exists in a stable form that is not solubilized in hypotonic medium. Lipase, trypsin, Triton 100, and freeze-thaw treatment all failed to solubilize the enzyme. N-butyl alcohol, which solubilizes alkaline phosphatase, inactivated the acid phosphatase. Further purification of the cream enzyme was, therefore, unsuccessful.

4. *Preparation of acid phosphatase from skimmilk.* Results of centrifugation studies (Table 5) showed that large amounts of acid phosphatase remain in the supernatant of skimmilk centrifuged at 37,500 G for 60 min. (2,250 × 10^3 G × min.). This is in contrast to buttermilk in which the enzyme is almost completely sedimented. The obvious discrepancies in percentages reported in Table 5 are due to the difficulty in accurately assaying raw skimmilk. After

TABLE 5

Acid phosphatase [a] distribution in differential centrifugation of buttermilk and skimmilk

	Buttermilk		Skimmilk	
	Units/ml	Per cent	Units/ml	Per cent
Original material	.400	100	.370	100
Centrifuge 150 × 10^3 G × min.				
Supernatant	.421	105.2	.394	106.5
Sediment	.057	14.3	.242	65.5
Supernatant recentrifuged 2,250 × 10^3 G × min.				
Supernatant	.061	15.2	.338	91.5
Sediment	.366	91.4	.382	103.5

[a] Determined with disodium phenyl phosphate substrate.

dialyzing against 0.15 *M* NaCl for 24 hr. and 0.25 *M* sucrose for 15 hr., the enzyme activity was almost doubled. This is probably due to the presence of inhibitory substances in the raw skimmilk.

Figure 2 outlines the procedure for separating acid phosphatase from skimmilk. Although activity was present in both the supernatant and sediment after the initial centrifuging at 50,000 G for 1 hr., only the supernatant was retained for further purification. The yield of acid phosphatase in acid whey was better

than in rennet whey. A similar observation has been made by others (1). The soluble acid phosphatase recovered in the final step after dialyzing against distilled water had a specific activity of 0.36 as compared to 0.0121 for raw skimmilk. This represents a threefold purification. Greater purification can probably be achieved through salt fractionation and column chromatography techniques. Such work is under way and results will be reported in a subsequent paper.

SOME PROPERTIES OF PARTIALLY PURIFIED ACID PHOSPHATASES

The above experiments show the existence of two types of acid phosphatase—a particulate enzyme in cream and a soluble enzyme in skimmilk. The properties of the two enzyme preparations were compared.

1. *pH activity curve.* The optimum pH for the enzymes from both sources was determined using 0.1 *M tris*-maleate buffer (1a) and disodium *p*-nitrophenyl phosphate substrate. Figure 3 shows a pH optimum in the vicinity of pH 5.0 for the cream enzyme. A broad pH optimum in the same range was obtained with the skimmilk preparation. With 0.1 *M* succinate-borate buffer and 0.1 *M* acetate buffer, similar results with a more pronounced peak were obtained. The pH optimum is 4.0 when disodium phenyl phosphate substrate and succinate-borate buffer are used. In the presence of 0.01 *M* sodium fluoride, acid phos-

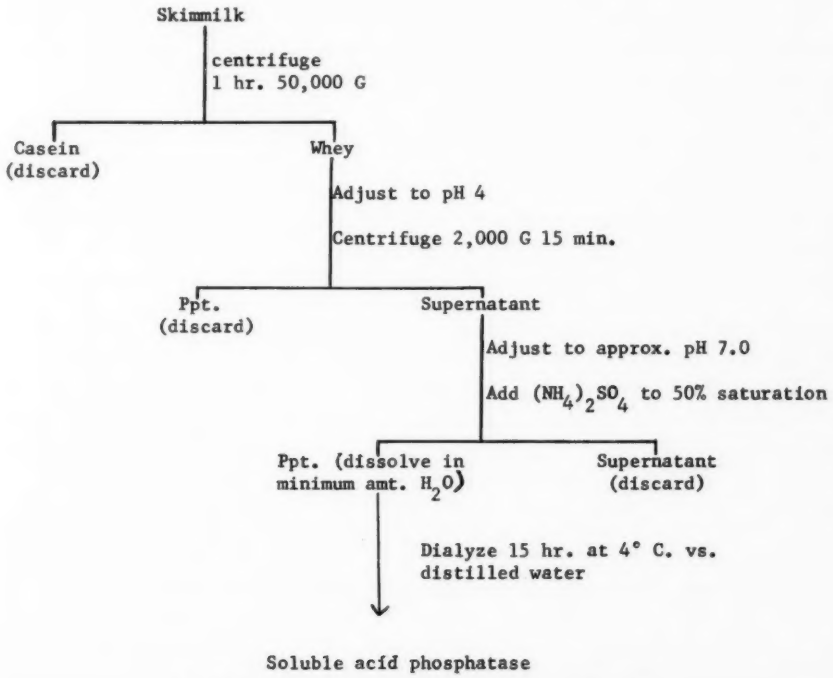

FIG. 2. Preparation of acid phosphatase from skimmilk.

phatase is inhibited but alkaline phosphatase (pH 8.2 and higher) is not affected.

2. *Michaelis constant.* Constants were determined for the two enzymes, using a broad range of concentrations of disodium *p*-nitrophenyl phosphate substrate in 0.1 *M tris*-maleate buffer, pH 5.0. The Michaelis constant for the purified cream acid phosphatase was 0.0010 as compared to 0.00051 for the purified skimmilk acid phosphatase. Further purification of the enzyme is desirable before it can be concluded that the two enzymes differ in this respect. The value for the cream phosphatase was 0.0020 when phenyl phosphate was the substrate. Mullen (9) reported a Michaelis constant of 0.0032 for the acid phosphatase in whole milk with phenyl phosphate substrate.

3. *Effect of inhibitors.* In these studies the enzyme, inhibitor, and 0.2 *M* acetate buffer, pH 5.0, were mixed and allowed to react at 38° C. for 20 min. before adding disodium *p*-nitrophenyl phosphate substrate and timing the assay. Each enzyme was tested with at least three concentrations of inhibitor. Results were plotted as reciprocals of per cent activity versus the concentration of the

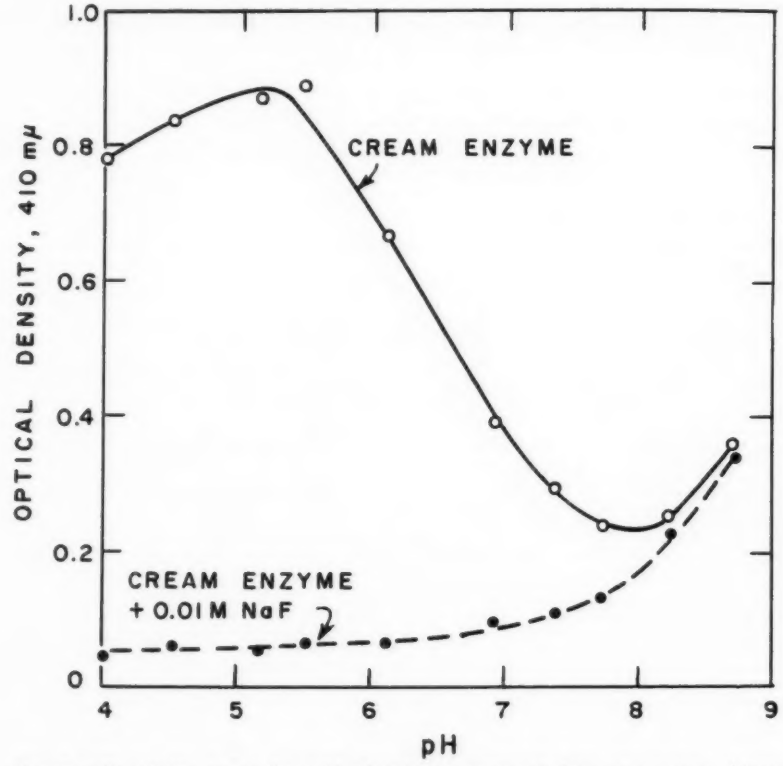

FIG. 3. pH activity curves for acid phosphatase prepared from raw cream. (Assayed with nitrophenyl phosphate substrate, with 0.1 *M tris*-maleate buffer.)

TABLE 6

Effect of inhibitors on acid phosphatases[a] prepared from raw skimmilk and raw cream

Inhibitor	50% inhibition molar concentration	
	Skimmilk enzyme	Cream enzyme
NaH_2PO_4	.005	.002
Na_3AsO_4	.005	.006
$CuSO_4$	>.001	.0007
$Hg(C_2H_3O_2)_2$	.0005	.0004
NaF	<.0001	<.0001

[a] Determined with disodium *p*-nitrophenyl phosphate substrate.

inhibitor. Table 6 lists some substances exerting inhibitory action and gives the molarity causing 50% inhibition. The cream enzyme was more affected by NaH_2PO_4 and $CuSO_4$ than was the skimmilk preparation. Among other reagents studied, KCN was slightly activating to the cream enzyme but did not affect the skimmilk enzyme. There were no marked effects from $MgCl_2$, $CaCl_2$, $FeCl_3$, l-tartrate, or Versene (disodium ethylene diaminetetraacetate). The inhibitory effects of NaF and $CuSO_4$ had been observed previously (1).

DISCUSSION

The determination of acid phosphatase in milk and cream was hindered by several difficulties which must be considered. The acid phosphatase content is low and relatively large aliquots were necessary for the assays. Because of this, the presence of alkaline phosphatase, which if about 50 times greater in amount, is a source of concern. The shape of the pH activity curve and the influence of sodium fluoride, which inhibits acid phosphatase but not alkaline phosphatase, indicates that alkaline phosphatase does not exert any activity at pH 4 to 5.

The large amount of protein precipitated in the assay mixture is troublesome. This is particularly true in assaying skimmilk with phenyl phosphate substrate at pH 4.0. With nitrophenyl phosphate at pH 5.0, the precipitate is finer and more easily kept in a homogeneous suspension during the assay. The estimation of the acid phosphatase in milk has been investigated in detail (4a).

The results show also that direct assays of milk and cream are limited by the presence of an inhibitor, presumably phosphate, in view of the strong inhibition of the purified phosphatases by phosphate. To avoid this inhibition, samples of milk and cream for assay may be dialyzed. The inhibitor is not present in the purified samples. Phosphate is a stronger inhibitor of acid phosphatase than of alkaline phosphatase (11).

The pH optimum reported by Mullen (9) has been confirmed for this phosphatase, and the Michaelis constant is of the same magnitude as that found by Mullen. The marked resistance of this enzyme to heat has also been confirmed. It has been found further that this resistance lessens as the phosphatase is separated from other proteins.

The ultracentrifuge experiments with the cream preparation show that the acid phosphatase is easily sedimented and probably is associated with the fat globule membrane. The distribution of acid phosphatase, alkaline phosphatase,

and xanthine oxidase indicate that the fat globule membrane fraction is not homogeneous but has a broad spectrum of size and density. The ultracentrifuge experiments also indicate that the acid phosphatase in skimmilk does not sediment readily and is presumably dissociated from the lipoprotein complex. It does not, however, appear to be in equilibrium with the complex in the cream, since the cream complex resisted efforts to dissociate it. The dissociated form of the acid phosphatase in skimmilk seems to be a likely form for purification and attempts along this line appear to be promising.

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SEASONAL CHANGES IN INTERRELATIONSHIPS AMONG MILK CONSTITUENTS [1]

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SUMMARY

Since total soluble phosphorus, soluble organic and inorganic phosphorus, citric acid, and magnesium show important seasonal changes, differences in the relationships of these constituents to other milk constituents have been studied. Important changes in correlation between constituents are shown to occur with changes in season.

The correlation coefficients between total soluble phosphorus and many other milk constituents change from highly significant to not significant with changes in season. The data show marked changes in relationships with casein, calcium, and phosphorus. The relation between soluble inorganic phosphorus and the soluble calcium also shows major shifts with changes in season. The magnitude of these shifts is sufficiently great that changes in stability of the milk would be expected.

The soluble organic phosphorus is not closely associated with any of the major milk constituents. There is little evidence to suggest that changes in this constituent could account for seasonal changes in the properties of milk.

Citric acid is normally quite independent of the other milk constituents. However, in the spring it is highly significantly correlated with casein, colloidal phosphorus, and the insoluble calcium. Association of citrate with the casein micelles and the colloidal calcium phosphate appear to be seasonal phenomena. Magnesium also shows its most significant relationships in the spring. In other seasons, its changes are independent of the other major constituents. No relationship between magnesium and soluble calcium is indicated at any season.

The chemical composition of 258 bulk milk samples and general interrelationships among the constituents have been reported (2, 3). Nearly all of the constituents vary significantly in quantity from season to season. The few exceptions are: proteose-peptone N, nonprotein N, total organic P, insoluble organic P, and soluble Ca. The other milk constituents vary not only in amount but also in relation to each other. Little work has been done in this area. Such variations are reflected in changes in salt balance (5), and presumably in processing or stability problems, although the latter lacks conclusive evidence.

Reported herein are results of statistical analyses of seasonal changes in the composition of milk from six areas of California. The quantity of most milk constituents normally varies more with the source of the milk (area, breed, feed, etc.) than with the season. The exceptions showing greater changes with season are total soluble phosphorus, soluble organic and inorganic phosphorus, citric acid, and magnesium. It seemed likely that these constituents might be associated with the seasonal changes in milk that result in processing difficulties. Therefore, their correlations with the other constituents were analyzed to determine any significant changes related to season.

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Total soluble phosphorus. Correlation of total soluble phosphorus with a number of the other milk constituents changes with season from highly significant to not significant. The data in Table 1 show this wide variation in association with total solids, fat, solids-not-fat, casein N, soluble calcium, and insoluble organic P. These constituents tend to show high correlations in the spring (February, March, April). At that time, there is a close relationship between the amount of soluble phosphorus and the amounts of these other constituents, but as the year progresses the relation changes and the constituents vary independently. The data illustrate particularly the marked changes in relationships with casein, calcium, and phosphorus. Such changes would be expected to have a pronounced effect on the stability of the calcium caseinate–phosphate complex.

Soluble inorganic phosphorus. The inorganic phosphorus in solution is in equilibrium with the colloidally dispersed phosphates and influences the stability of the caseinate system. Variations of this constituent in relation to the others are, therefore, considered important to the physical properties of the milk.

The data in Table 2 show changes in significance of the correlation coefficients with the other milk constituents. The soluble inorganic phosphorus is significantly correlated with the solids and casein, except in the winter (IV, November, December, January). Also during the winter, it is interesting to note that the soluble inorganic phosphorus is significantly correlated only to soluble calcium among the major milk constituents. The relation between soluble phosphorus and soluble calcium also shows major shifts with changes in season. Changes in soluble phosphorus relative to the other major constituents, as shown in these data, are great enough that stability changes would be expected.

Soluble organic phosphorus. The data in Table 3 show that soluble organic phosphorus is at no season closely associated with any of the major milk constituents. The significant relationships are primarily with other forms of phosphorus. Negative correlation between soluble organic and soluble inorganic phosphorus is highly significant in the spring, of decreasing significance during the summer, and without significance in fall and winter. There is little evidence here to suggest that changes in soluble organic phosphorus are important in causing seasonal changes in milk.

Citric acid. Most of the time, citric acid is quite independent of the other milk constituents. Nevertheless, the data in Table 4 indicate some very interesting relationships, particularly in the spring. At that time, there are highly significant correlations with casein, colloidal phosphorus, and the insoluble calcium. This indicates that the reported association of citrate with the casein micelles (1) and inclusion of citrate in the colloidal calcium phosphate (4) are likely to be seasonal phenomena. During the other seasons, these relationships are not apparent in our data. The data also suggest that the citrate associated with the micelles may be independent of those associated with the colloidal calcium phosphate. Evidence is that in May, June, and July citric acid is significantly correlated with casein, but not with the colloidal phosphorus or insoluble calcium.

TABLE 1

Significance by seasons of correlation coefficients between total soluble phosphorus and other milk constituents

	Highly significant				Significant				Not significant			
	Season				Season				Season			
	I[a]	II[b]	III[c]	IV[d]	I	II	III	IV	I	II	III	IV
1. Total solids	.402						.310			.119		−.066
2. Fat	.352									.029	.267	−.101
3. S.N.F.	.400									.204	.276	.051
4. Total nitrogen	.380									.125	.208	−.101
5. Casein N	.376									.099	.182	−.104
6. Lactalbumin and lactalbumin N									.195	.191	.172	−.162
7. Proteose-peptone N									.114	−.065	.287	−.023
8. Nonprotein N									.008	.151	−.084	.144
9. Total phosphorus	.675	.554		.305			.367					
11. Total inorganic P	.474	.385						.279			.271	
12. Soluble inorganic P	.403	.428	.422	.570								
13. Total organic P	.459	.408									.218	.087
14. Soluble organic P	.630	.717	.858	.769								
15. Insoluble organic P			−.435	−.385					−.007	−.061		
16. Colloidal inorganic P					.253					.198	.055	−.010
17. Total insoluble P				−.389			−.367		.218	.087		
18. Citric acid						−.295			.220		−.121	.071
19. Total Ca + Mg	.335					.264					.107	−.153
20. Total Ca	.456									.096	.256	−.118
21. Total Mg						.281			.016		−.102	−.092
22. Soluble Ca	.454									.003	.220	.102
23. Insoluble Ca					.257					.093	.184	−.203

[a] February, March, and April; [b] May, June, and July; [c] August, September, and October; [d] November, December, and January.

TABLE 2

Significance by seasons of correlation coefficients between soluble inorganic phosphorus and other milk constituents

	Highly significant				Significant				Not significant			
	Season				Season				Season			
	I[a]	II[b]	III[c]	IV[d]	I	II	III	IV	I	II	III	IV
1. Total solids		.403	.403		.262							−.029
2. Fat		.389			.234		.348					−.038
3. S.N.F.					.250	.282	.355					.007
4. Total nitrogen		.387			.255		.348					.135
5. Casein N		.378			.275		.365					.114
6. Lactalbumin and lactalbumin N									.021	.037	−.017	−.050
7. Proteose-peptone N							.355		.093	.178		.136
8. Nonprotein N								.292	.024	.038	.005	
9. Total phosphorus	.459	.524					.298					.168
10. Total soluble phosphorus	.403	.428	.422	.570								
11. Total inorganic P	.438	.532	.457									.220
13. Total organic P									.191	.216	−.032	.012
14. Soluble organic P	−.457	−.322									−.105	−.087
15. Insoluble organic P	.499	.410									.055	.022
16. Colloidal inorganic P								−.283	−.180	.030	−.107	
17. Total Insoluble P	.336	.380									−.012	−.227
18. Citric acid									.113	−.017	.059	−.056
19. Total Ca + Mg		.386			.250						.195	−.039
20. Total Ca	.433	.437									.252	−.073
21. Total Mg									−.064	.036	.011	.040
22. Soluble Ca	.448		.563	.317						.026		
23. Insoluble Ca		.416			.234			−.286			−.061	

[a] February, March, and April; [b] May, June, and July; [c] August, September, and October; [d] November, December, and January.

TABLE 3

Significance by seasons of correlation coefficients between soluble organic phosphorus and other milk constituents

	Highly significant				Significant				Not significant			
	Season				Season				Season			
	I[a]	II[b]	III[c]	IV[d]	I	II	III	IV	I	II	III	IV
1. Total solids									.169	−.186	.111	−.058
2. Fat						−.270			.143		.095	−.093
3. S.N.F.									.176	−.004	.101	.056
4. Total nitrogen								−.228	.153	−.167	.031	
5. Casein N									.132	−.188	−.008	−.215
6. Lactalbumin and lactalbumin N									.172	.172	.198	−.157
7. Proteose-peptone N									.032	−.205	.113	−.133
8. Nonprotein N									−.012	.128	−.095	−.052
9. Total phosphorus					.266			.239		.177	.233	
10. Total soluble phosphorus	.630	.717	.858	.769								
11. Total inorganic P									.088	−.007	.039	.167
12. Soluble inorganic P	−.457	−.322									−.105	−.087
13. Total organic P					.283	.260					.256	.096
15. Insoluble organic P	−.431	−.380	−.508	−.483								
16. Colloidal inorganic P	.398									.184	.121	.207
17. Total insoluble P			−.396					−.295	−.073	−.201		
18. Citric acid						−.295			.118		−.166	.129
19. Total Ca + Mg									.113	−.022	.006	−.155
20. Total Ca									.076	−.236	.138	−.086
21. Total Mg						.267			.070		−.118	−.142
22. Soluble Ca									.061	−.016	−.078	−.123
23. Insoluble Ca									.051	−.223	.237	−.023

[a] February, March, and April; [b] May, June, and July; [c] August, September, and October; [d] November, December, and January.

TABLE 4

Significance by seasons of correlation coefficients between citric acid and other milk constituents

	Highly significant				Significant				Not significant			
	Season				Season				Season			
	I[a]	II[b]	III[c]	IV[d]	I	II	III	IV	I	II	III	IV
1. Total solids					.249					.175	−.209	−.021
2. Fat					.248					.173	−.149	−.004
3. S.N.F.									.192	.118	−.268	−.053
4. Total nitrogen					.281	.304					−.258	−.182
5. Casein N	.296					.309					−.228	−.147
6. Lactalbumin and lactalbumin N									.187	.195	−.202	−.102
7. Proteose-peptone N									−.002	.068	−.149	−.159
8. Nonprotein N						−.249			−.154		−.170	−.212
9. Total phosphorus					.295					−.011	−.124	−.028
10. Total soluble phosphorus						−.295			.220		−.121	.071
11. Total inorganic P	.371									.070	.146	−.030
12. Soluble inorganic P									.113	−.017	.059	−.056
13. Total organic P									.029	−.086	−.283	−.044
14. Soluble organic P						−.295			.118		−.166	.129
15. Insoluble organic P									−.055	.103	−.127	−.075
16. Colloidal inorganic P	.332									.092	.133	−.002
17. Total insoluble P					.241					.156	−.035	−.076
19. Total Ca + Mg									.110	.126	.024	−.092
20. Total Ca	.339									.235	−.079	.012
21. Total Mg									−.150	−.106	.103	−.177
22. Soluble Ca									−.046	.124	−.048	−.054
23. Insoluble Ca	.360									.162	−.071	.049

[a] February, March, and April; [b] May, June, and July; [c] August, September, and October; [d] November, December, and January.

TABLE 5

Significance by seasons of correlation coefficients between magnesium and other milk constituents

	Highly significant				Significant				Not significant			
	Season				Season				Season			
	I[a]	II[b]	III[c]	IV[d]	I	II	III	IV	I	II	III	IV
1. Total solids									−.108	.074	−.150	.126
2. Fat									−.138	−.001	−.183	.124
3. S.N.F.									−.025	.153	.005	.071
4. Total nitrogen					−.296					−.067	−.169	.148
5. Casein N					−.258					−.061	−.133	.131
6. Lactalbumin and lactalbumin N									−.188	−.124	−.062	.124
7. Proteose-peptone N							−.374		−.108	−.035		.006
8. Nonprotein N					−.239					.120	−.063	.172
9. Total phosphorus									.015	.180	−.013	.209
10. Total soluble phosphorus						.281			.016		−.102	−.092
11. Total inorganic P	−.321									.073	.089	.084
12. Soluble inorganic P									−.064	.036	.011	.040
13. Total organic P	.356									.184	−.095	.159
14. Soluble organic P						.267			.070		−.118	−.142
15. Insoluble organic P					.287					.009	.001	.212
16. Colloidal inorganic P	−.309									.065	.100	.062
17. Total insoluble P								.265	.009	.054	.062	
18. Citric acid									−.150	−.106	.103	−.177
19. Total Ca + Mg	.722	.564	.683	.540								
20. Total Ca									−.191	−.107	−.170	−.046
22. Soluble Ca									.019	−.208	.070	−.136
23. Insoluble Ca					−.228					.010	−.274	.032

[a] February, March, and April; [b] May, June, and July; [c] August, September, and October; [d] November, December, and January.

Magnesium. Magnesium also shows its most significant relationships with other milk constituents in the spring. In other seasons, it is essentially independent (Table 5). In the spring, magnesium varies inversely with casein, nonprotein N, colloidal inorganic phosphorus, and insoluble calcium. This is additional statistical evidence of no constant ratio of calcium and magnesium with casein. Nor is there appreciable magnesium in the colloidal phosphates. The data indicate no relation between magnesium and soluble calcium at any season.

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EFFECT OF pH AND BRINE CONCENTRATION ON GAS PRODUCTION BY A PUTREFACTIVE ANAEROBE (PA) 3679 IN A PROCESSED CHEESE SPREAD [1, 2]

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SUMMARY

Gas production by PA (putrefactive anaerobe) 3679 in a processed cheese spread containing 8.5% skimmilk powder and packaged in TDT (thermal death time) cans incubated at 37° C. was studied at pH levels of 5.2 to 7.0 and brine concentrations of 2.0 to 8.4%. Most of the expansion attributable to gas production in the TDT cans occurred during the first 20 days and no measurable gas production developed after 60 days. As the pH decreased from 7.0 to 6.0, the lag time for gas production increased and gas production was completely inhibited at pH 5.6 and below. As the brine concentration increased, the lag time for gas production increased, and there was a decrease in the rate, total amount, and duration of gas production. Gas production was completely inhibited at a brine concentration of 7.6% or more.

A technique using a dial-micrometer was developed for detecting small amounts of gas production in TDT cans.

The temperature of approximately 70° C. attained in the manufacture of processed cheese spreads is sufficiently high to accomplish blending, emulsification, partial inactivation of natural and microbial enzymes, and destruction of most of the nonheat-resistant microorganisms. However, the prevention of spoilage of the product by thermoduric organisms is dependent upon several interrelated inherent properties which are controlled by the composition of the product.

The study reported herein was conducted to determine the effect of two of these properties (pH and brine concentration) on the gas production of a typical heat-resistant food spoilage organism, PA (putrefactive anaerobe) 3679, in a processed cheese spread.

EXPERIMENTAL METHODS

A culture of PA 3679 was obtained from the Wyeth Laboratories, Inc., Mason, Michigan, and a spore suspension was prepared according to the method of Reed *et al.* (5) and harvested by the procedure of Townsend *et al.* (10). The washed spore suspension was standardized by determining the most probable number (MPN) in the concentrated suspension and then diluting with neutral *M*/15 phosphate buffer to obtain approximately 10^6 spores per milliliter. The MPN was determined, using five tubes of liver infusion broth per dilution, pre-

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pared according to the method of Reed *et al.* (5). The tubes were stratified with a mixture of two parts mineral oil to one part paraffin and exhausted for 20 min. in flowing steam prior to inoculation.

The tubes were then tempered to 45° C. in a water bath, inoculated with a proper dilution of spores, heated for 10 min. at 80° C. to heat-shock the spores, and incubated at 37° C. A preliminary estimation of MPN was made by observing for gas production at the end of 2 wk.; however, the final count was determined from the results obtained after incubation for 3 mo.

A processed cheese spread was prepared by combining 43 lb. of aged Cheddar, 30 lb. of unsalted green Cheddar curd, 22 lb. of water, 10 lb. of 35% cream, 10 lb. of skimmilk powder, and 3 lb. of sodium citrate. This mixture was heated to 71° C. in a Mojonnier processing kettle; the finished product was drawn into No. 10 cans (603 × 700)[4] and the cans sealed. The cans of processed cheese spread were then cooled and stored in a 40° F. refrigerator. Analysis of this cheese showed 46.04% moisture, 22.34% fat, 0.775% salt, and a pH of 6.20. The brine concentration was 1.65%. The fat and moisture were determined by the Mojonnier method (3) and the method of Wilster *et al.* (15) was used to determine the salt content.

The substrate for each test was prepared from the canned processed cheese base. A sealed can was removed from the refrigerator, allowed to temper overnight at room temperature, then placed in a covered water bath at 65° C. for one-half day. The can was opened and the required amount of processed cheese spread weighed into a beaker, which was placed in a 65° C. water bath. A calculated volume of the standardized spore suspension was added to give 1.84×10^5 spores per gram of cheese. The brine concentration was standardized by adding the amount of salt (NaCl) needed as calculated by the following equation:

$$\text{grams of salt} = \frac{\text{desired brine concentration (per cent)} \times \text{grams water}}{100 - \text{desired brine concentration (per cent)}}$$

Appropriate corrections were made for the salt originally in the cheese and the water added with the spore inoculum.

The grams of water added with the spore suspension and the salt present in the processed cheese spread were taken into consideration when standardizing the brine concentration. The pH was adjusted by adding 6 *N* HCl or NaOH as required. All pH determinations were made at 65° C., using both a glass and a quinhydrone electrode. Gas production tests were conducted both in test tubes and in thermal death time (TDT) cans (208 × 006).[5] The inoculated standardized processed cheese spread was dispensed into test tubes using a 2-oz. syringe, modified by the addition of a metal tube (0.7 cm. inside diameter and 15 cm. long). Each tube was stratified using a paraffin mineral oil mixture, plugged, heated for 10 min. at 80° C. to heat-shock the spores, tempered to

[4] 6 3/16 in. in diameter and 7 in. high.

[5] 2 8/16 in. in diameter and 6/16 in. high.

37° C., and incubated at 37° C. TDT cans were filled with 19 g. of the inoculated standardized processed cheese spread, sealed, heated for 10 min. at 80° C., tempered, and incubated at 37° C.

The processed cheese spread was not sterilized prior to inoculation, because a severe heat treatment would have altered the product; however, it was heated to 71° C. at time of processing, to 65° C. for at least 4 hr. prior to standardization, and to 80° C. for 10 min. after dispensing into tubes and cans. No determination was made of the microbiological content of the cheese prior to inoculation, but TDT cans of uninoculated cheese spread incubated at 37° C. showed no expansion.

The test tubes of inoculated processed cheese spread were checked visually for evidence of gas bubbles. Initially, the expansion of the TDT cans of processed cheese spread was determined visually; however, this was not satisfactory. A technique was developed in which a standard dial-micrometer gauge (Figure 1) was used to measure vertical expansion of TDT cans within ± 0.0005 in. All measurements were made in a constant temperature (37° C.) walk-in incubator to avoid changes in thickness of cans due to temperature fluctuation.

RESULTS

The results of a test to determine the effect of pH and brine concentration on the ability of PA 3679 to produce gas in processed cheese spread are shown by the data in Table 1. There was gas production above and no gas production at or below pH 5.6 throughout the range of brine concentration tested (2.8 to 6.8%).

TABLE 1

Effect of pH and brine concentration on the gas production of PA 3679 in cheese spread incubated 14 days at 37° C. (measured by gas production in glass tubes, one tube per test variable)

	Brine concentration (%)					
pH	2.8	3.6	4.4	5.2	6.0	6.8
5.2	−	−	−	−	−	−
5.4	−	−	−	−	−	−
5.6	−	−	−	−	−	−
5.8	+	+	+	+	+	+
6.0	+	+	+	+	+	+
6.2	+	+	+	+	+	+

In another test, in which both tubes and TDT cans were used (Table 2), the gas-producing ability of PA 3679 in tubes of processed cheese spread with a pH of 6.2 was completely inhibited at a brine concentration equal to or greater than 7.6%. The small amount of gas produced at brine concentrations of 2.8 to 6.8% did not cause enough expansion of TDT cans to be visually detectable. The dial-micrometer technique, previously mentioned, was developed for measuring a small amount of TDT can expansion.

The data in Table 3 show the effect of pH and brine concentration on the lag time for gas production by PA 3679 in processed cheese spread.

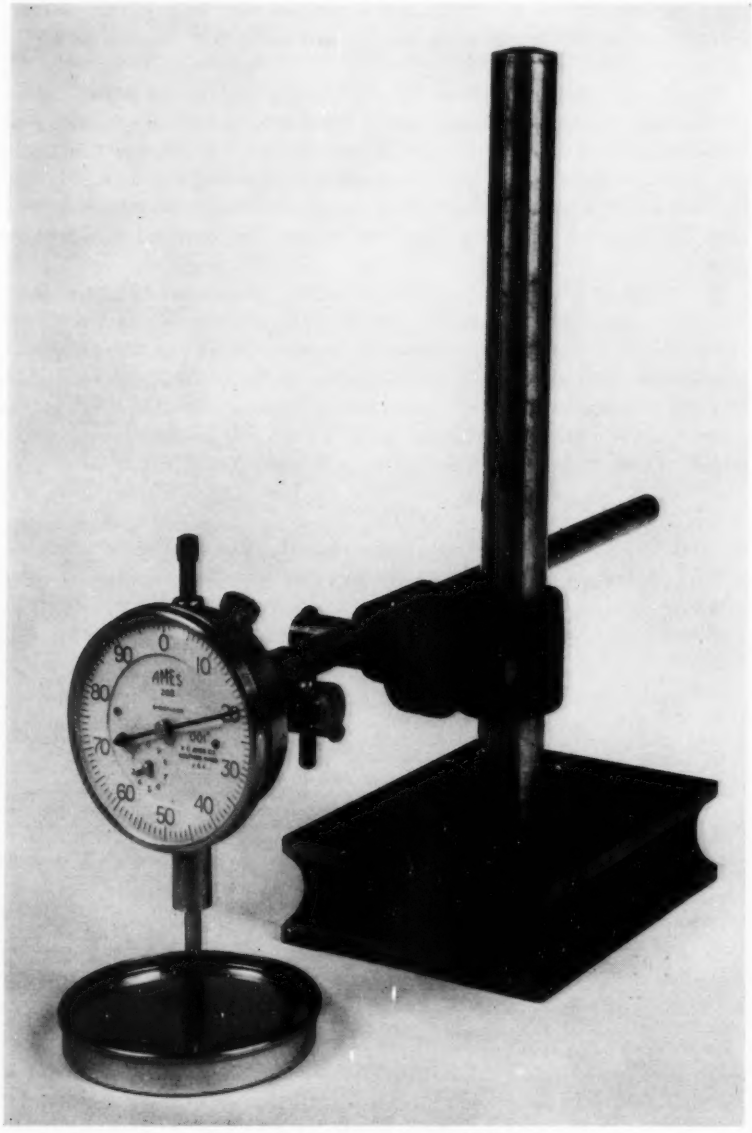

FIG. 1. Dial-micrometer and TDT can.

Values showing the final amount of total expansion of the cans of cheese at all eight brine concentrations at pH 6.0 and 7.0 after 60 days of incubation are shown in Figure 2. An increase in the brine concentration resulted in an

TABLE 2

Effect of brine concentration on the gas production of PA 3679 in cheese spread with a pH of 6.2 when incubated 14 days at 37° C. (five tubes and one TDT can per test condition)

Brine concentration (%)	Visual examination	
	Glass tubes	TDT cans
2.0	+++++	+
2.8	+++	−
3.6	++	−
4.4	++	−
5.2	+	−
6.0	+	−
6.8	+	−
7.6	−	−
8.4	−	−

increase in the lag time for gas production, a decrease in the total amount of gas production, and a decrease in the duration of time during which gas was produced. There was no measurable gas production after 60 days of incubation.

DISCUSSION

Gas production by PA 3679 was completely inhibited at or below a pH of 5.6. Most investigators (4, 7-9) agree that decreasing the pH of processed cheese spread extends the keeping quality. Hood and Smith (2) and Ritchie (6) reported that putrefactive spoilage is completely eliminated at a pH of less than 5.4. In working with a surface-ripened cheese containing stabilizer, Wagenaar and Dack (13) found more growth and toxin production by *Clostridium botulinum* when the pH of the cheese was above 5.7 than when the pH was 5.7 or lower. The acidification of the processed cheese used in this project to a pH of less than 5.7 resulted in an objectionably crumbly body and texture.

Each increase in the brine concentration of the product resulted in an increased lag time for gas production, a decreased rate of gas production, and a decreased amount of gas production. Gas production by PA 3679 was completely inhibited at a brine concentration of 7.6% or greater. Wagenaar and Dack (12-14) encountered growth and toxin production by *C. botulinum* in cheese with a brine concentration of 6 to 8%.

TABLE 3

Effect of pH and brine concentration on the lag time for gas production by PA 3679 in a cheese spread incubated at 37° C. (measured by the swelling of TDT cans, ten cans per test variable)

Brine concentration (%)	Lag time in days	
	pH 6.0	pH 7.0
1.6	4	3
2.0	4	3
2.4	6	4
2.8	6	4
3.2	7	6
3.6	10	7
4.0	12	10
4.4	15	12

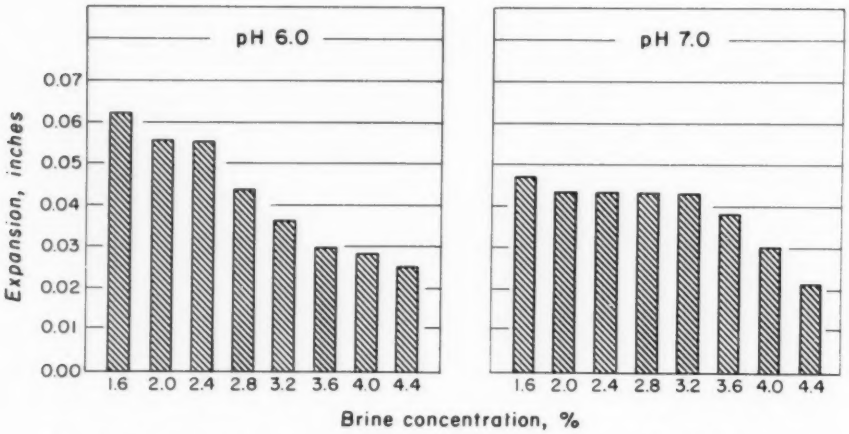

FIG. 2. Effect of brine concentration on the gas production of PA 3679 in a cheese spread at pH 6.0 and 7.0, as measured by the expansion of TDT cans incubated 60 days at 37° C. (each bar represents an average of ten cans).

The dial-micrometer technique for measuring expansion in TDT cans is a new approach to the problem of detecting gas production of certain anaerobic microorganisms in a partially inhibitory growth medium. The technique has the following limitations: (a) Gas production can not be quantitatively or qualitatively related to growth, (b) apparent gas production will vary with the pH and chemical composition of the substrate, and (c) rate and amount of expansion of cans does not necessarily vary linearly with gas production but is a function of the solubility of the gas, the initial fill of the container, and the initial pressure within the container. Data obtained using this technique may be interpreted in the following ways: (a) Expansion of cans is attributed to gas production resulting from metabolic activity of the organisms, (b) the time necessary to initiate expansion of cans is the lag phase of detectable gas production, (c) the cessation of expansion may be interpreted as the termination of gas production, and (d) gas production at different brine concentrations may be compared as long as other factors remain constant, since the effect of brine concentration on the solubility of gas is negligible. The dial-micrometer technique has several advantages: (a) relatively simple, (b) a large number of replications can be carried out without severely overtaxing facilities, (c) the tests are nondestructive, so that one set of samples can be followed through an entire cycle, and (d) the experiment can be carried out under actual incubation conditions for the product.

This study has evaluated the effect of pH and brine concentration for only one processed cheese formulation. A preliminary test carried out as part of this study indicated that the amount of skimmilk powder used in the processed cheese formulation affects the ability of PA 3679 to produce gas in the product.

Other workers (1, 6, 11) have made similar observations; therefore, to completely establish the relationship of pH and brine concentration in cheese spreads, future studies should evaluate in detail the effect of varying the processed cheese formulation.

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STAPHYLOCOCCI IN COLBY CHEESE [1]

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SUMMARY

Colby cheese was manufactured from raw milk inoculated with a coagulase-positive *Staphylococcus aureus* and from noninoculated raw milk obtained from cows with subclinical mastitis. Organisms were enumerated on plate count agar (PCA) and on Staphylococcus Medium No. 110 (S-110) on samples of milk, curd, and whey taken during the cheese-making process and on samples of cheese taken at 15-day intervals during storage for 4 mo. at 10° C. Many representative colonies were picked from S-110 medium and examined for coagulase reaction.

At the time the curd was cut, the counts on S-110 medium were 13 to 32 times greater in the curd than in the whey. Between cutting and hooping the curd, the populations on S-110 medium increased 6.5 to 29 times, with the greatest increase occurring in the cheese made from the mastitic milk. The maximum populations on S-110 medium occurred in the curd when hooped or in the cheese when one day old and were high enough to cause the cheese to be a potential source of clinical levels of enterotoxin, regardless of decreases in populations during subsequent ripening.

As the aging period progressed the percentage of coagulase positive colonies picked from S-110 medium gradually declined, but not uniformly, and coagulase-positive colonies were obtained from cheese cured for 120 days at 10° C. At the end of the 120-day holding period the populations on S-110 medium varied from 0.07 to 1.7% of the corresponding counts on the first day and the highest percentage of surviving organisms occurred in the cheese made from the mastitic milk. These results, together with the greater multiplication previously mentioned, indicate that the staphylococci naturally present in milk are better adapted to the environment of the cheese-making and curing process than the laboratory cultures of *S. aureus* which were inoculated into the milk.

In recent years, the United States Public Health Service has reported numerous cases of food poisoning traced to cheese (2, 3, 4, 5) and several detailed reports have appeared in the literature. Hendricks *et al.* (9), Hausler *et al.* (7), Legler *et al.* (11), Neuman *et al.* (14), and McDonald (13) investigated outbreaks of food poisoning involving cheese and found the causative organisms to be *Staphylococcus aureus.*

Thatcher *et al.* (16) surveyed Canadian cheese in 1956 and found that 45% of the raw milk cheese and 13% of the pasteurized milk cheese contained coagulase-positive *S. aureus.* In 1959, Takahashi and Johns (15) found *S. aureus* in excess of 10,000 per gram in 119 out of 333 phosphatase-positive samples of Canadian Cheddar Cheese.

Heineman (8) studied the growth and thermal destruction of *Micrococcus pyogenes* var. *aureus (S. aureus)* in raw, pasteurized, and condensed skimmilks preheated to 205° F. (96° C.) for 20 min. prior to condensing. The milks were inoculated and incubated at a series of temperatures between 80 and 115° F. (27 to 46° C.). *S. aureus* grew poorly in raw milk at 27° C., but good growth

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occurred in the heat-treated condensed milks. A related phase of work established the thermal death time of *M. pyogenes* var. *aureus* (Culture 196E) as 1.9 min. at 150° F. (65.6° C.) when inoculated into skimmilk. However, Takahashi and Johns (15) reported that flash heating of milk to 65.6° C. effectively destroyed the staphylococci that were naturally present. Gibson and Abd-El-Malek (6) found that in normal raw milk the majority of the species of staphylococci are coagulase-negative and heat-sensitive. Jones *et al.* (10) studied the growth of *S. aureus* in milk obtained from two sources: (a) cows with a natural staphylococcal infection; and (b) aseptically drawn from healthy cows. The aseptically drawn milk was inoculated with *S. aureus*. Good growth was obtained in both milks at 30 and 37° C., but below 25° C. growth was extremely slow. Takahashi and Johns (15) found that milk with a low standard plate count (33,000 to 260,000 per milliliter) would support active growth of *S. aureus* at 32° C. and limited growth at 22° C., but in high-count milk containing 5,100,000 to 5,500,000 organisms per milliliter growth was very limited, even at 32° C.

Mattick *et al.* (12) made cheese with pasteurized milk obtained from cows with subclinical staphylococcal mastitis and inoculated with two strains of *S. aureus*. After 14 to 22 wk. of storage staphylococci could not be recovered, even with enrichment methods, and the cheese was consumed at 6 mo. with no ill effects. Takahashi and Johns (15) showed that staphylococci increased during Cheddar Cheese making and tended to concentrate in the curd. According to these investigators, milk with a high staphylococcus population will yield cheese with a high staphylococcus population, and greater numbers of staphylococci will survive in cheese made with milk that has a low initial total count.

Colby Cheese, with its high moisture, soft body, and open texture, appears to be a good environment for the growth of enterotoxigenic *S. aureus*. Since a large amount of Colby Cheese is made from raw milk, and significant numbers of *S. aureus* have been found in raw milk, it seemed important to determine the trend of population and length of time these organisms would survive in Colby Cheese.

METHODS AND MATERIALS

Thirteen vats of Colby Cheese were manufactured, using 100 lb. of raw milk in each vat. Seven vats of cheese (Group A) were made with good-quality raw milk procured from the University dairy plant. Four vats of cheese (Group B) were made with milk obtained from cows in the University herd which had subclinical mastitis and whose milk frequently contained coagulase-positive staphylococci. Two vats of cheese were made with milk that had been aged to simulate poor quality: one lot (Group C) at 25° C. for 16 hr., and one lot (Group D) at 1° C. for approximately three days.

The commercial method outlined by Wilster (18) was followed in the manufacture of the cheese, with the exception that immediately prior to adding rennet, each of the nine vats of nonmastitic milk (Groups A, C, and D) was inoculated with 13,000 to 500,000 *S. aureus* organisms per milliliter of milk. Four of the seven vats of milk in Group A were inoculated with *S. aureus* 161-C obtained from the R. A. Taft Sanitary Engineering Center, Cincinnati,

Ohio, and the other three were inoculated with *S. aureus* L16B obtained from the Department of National Health and Welfare, Ottawa, Canada. There was no significant difference between the results of analyses performed on the four vats inoculated with 161-C and the three vats inoculated with L16B; therefore, in reporting the data, the analyses of all seven vats were averaged together and included in Group A. The vats in Groups C and D were also inoculated with Strain L16B. During manufacture, samples were taken of the raw milk before and after inoculation with staphylococci, curd-when-cut, whey-at-cutting, whey-after-cooking, and curd-when-hooped. The cheese was stored at 10° C. and examined at one day, at approximately 15-day intervals during a period of 90 days, and at 120 days.

Microbiological populations were determined on curd and cheese samples by emulsifying a 25-g. portion with 50 ml. of sterile 2% sodium citrate for 2 min. in a sterile Waring Blendor jar equipped with a screw-cap. Plate count agar (Difco) was used for enumerating total populations and Staphylococcus Medium No. 110 (Difco) containing 0.004% acti-dione was used in the enumeration of staphylococci. The acti-dione was included to inhibit competitive growth of yeasts and molds and was effective for this purpose. Appropriate controls indicated that the above concentration of acti-dione was not inhibitory to *S. aureus*. The plates were incubated at 35° C. for 48 hr. In the performance of this work, it was deemed advisable to include in the enumeration all organisms growing on Staphylococcus Medium No. 110 (S-110) containing 0.004% acti-dione, because neither pigmentation, colonial morphology, nor any other known method of differentiation is consistently reliable in distinguishing staphylococci from other salt-tolerant organisms which occasionally appear on this medium.

At each examination, five or six representative colonies were picked from the staphylococcus medium into 2 ml. of brain heart infusion–mannitol broth as formulated by Wilson *et al.* (17). The cultures were incubated at 35° C. for 18 to 24 hr. Coagulase tests were made by inoculating 0.5 ml. of coagulase plasma (Difco) with two drops of the culture. The inoculated plasma was incubated at 35° C. and examined for coagulation at 1, 2, and 3 hr. A drop of each culture was Gram-stained and examined.

A modification of the enrichment method of Wilson *et al.* (17) was applied to the cheese–sodium citrate blends to determine whether there was any relationship between the population of organisms capable of growing on S-110 medium and the coagulase reaction of the enriched blend. One milliliter of brain heart infusion–mannitol salt broth was mixed with 0.5 ml. of the cheese–sodium citrate blend, incubated at 35° C. for 18 to 24 hr., and coagulase tests performed in the manner previously described.

At each examination a portion of the curd was macerated for pH determination.

RESULTS AND DISCUSSION

Changes in populations of organisms enumerated on plate count agar (PCA) and on S-110 medium during cheese manufacture. Data in Table 1 show the counts on PCA and on S-110 medium containing acti-dione on samples obtained

TABLE 1

Organisms enumerated on plate count agar and on Staphylococcus Medium No. 110 in samples taken during the manufacture of Colby Cheese [a]

Medium	*Staphylococcus aureus* culture	*S. aureus* per gram of milk	Description of sample: Noninoculated raw milk	Inoculated raw milk	Curd when cut	Whey at cutting	Whey at end of cooking	Curd when hooped
	GROUP A. Log. average of counts on seven vats of cheese made from good milk inoculated with *S. aureus*							
S-110	170,000,000	34,000	1,500	69,000	180,000	6,600	4,000	2,000,000
PCA	.....	.....	54,000	250,000	1,100,000	60,000	16,000	11,000,000
	GROUP B. Log. average of counts on four vats of cheese made from mastitic milk (not inoculated)							
S-110	.....	.....	5,600	.....	66,000	5,200	4,500	1,900,000
PCA	.....	.....	30,000	.....	1,000,000	150,000	74,000	9,100,000
	GROUP C. Counts on one vat of cheese made from milk aged 16 hr. at 25° C. and inoculated with *S. aureus*							
S-110	820,000,000	160,000	910,000	1,000,000	6,100,000	190,000	63,000	40,000,000
PCA	.....	.....	10,000,000	23,000,000	98,000,000	10,000,000	8,000,000	720,000,000
	GROUP D. Counts on one vat of cheese made from milk aged three days at 1° C. and inoculated with *S. aureus*							
S-110	680,000,000	130,000	<10,000	160,000	460,000	34,000	21,000	6,300,000
PCA	.....	.....	630,000	680,000	1,600,000	400,000	140,000	23,000,000

[a] Counts per milliliter or per gram.

during cheese manufacture. At the time the curd was cut, the counts on S-110 were from 13 to 32 times greater in the curd than in the whey, and corresponding counts on PCA varied from about four to 18 times greater in the curd than in the whey, which is normal. During the cooking operation, the S-110 population in the whey decreased from 14 to 67%, with the smallest decrease occurring in the whey from the mastitic (noninoculated) milk.

A comparison of the data from the cheese in Group A made with milk inoculated with *S. aureus* and Group B cheese, not inoculated, shows that between the time the curd was cut and the time it was hooped the counts on S-110 medium increased 11-fold and 29-fold, respectively. The total population increased tenfold in Group A cheese and ninefold in Group B cheese.

In the cheese made from inoculated milk (Groups A, C, and D) the populations determined on S-110 medium increased only three- to sixfold between the time the milk was inoculated and the curd was cut. These increases are not significant and represent concentration of the organisms in the curd portion. However, when the curd was hooped the population had increased 29- to 40-fold and increases of this magnitude must involve some multiplication.

The counts made on S-110 medium during the manufacture of the Group B cheese, made from noninoculated milk, showed a 12-fold increase from the raw milk to the time the curd was cut, and a 339-fold increase at the time the curd was hooped. Between cutting and hooping the curd the four groups of cheese showed a 6.5- to 29-fold increase in the population on S-110 medium, with the least increase occurring in the cheese made from milk aged 16 hr. at 25° C. (Group C) and the greatest increase occurring in the cheese made from the mastitic milk (Group B). The increases in population mentioned above are attributed to (a) affinity of the curd for organisms, (b) physical shrinkage of the curd because of whey exuded, and (c) growth, with the latter being the most important factor. Regardless of the source or quality of the milk, the maximum counts on S-110 medium were encountered either in the curd-when-hooped or in the cheese when one day old (Tables 1 and 2).

It is particularly noteworthy that when the four vats of cheese made from noninoculated milk (Group B) were one day old the logarithmic average of the counts on S-110 medium was 2,800,000 (Table 2), which is sufficient to render the cheese potentially dangerous as a source of enterotoxin, regardless of any subsequent decrease in population. Also, it is apparent from examining the counts in all four groups that cheese which may appear to be safe at 60 to 90 days may have previously possessed sufficient populations to produce enterotoxin.

Changes in populations of organisms enumerated in PCA and S-110 medium in cheese held at 10° C. for 120 days. Data on the bacteria counts performed during the period the cheese was stored at 10° C. are presented in Table 2. In general, the population on S-110 medium decreased as the age of the cheese increased, but minor exceptions occurred which may be attributed partially to inherent errors in sampling. During the first 15 days of the holding periods, the counts of organisms on S-110 decreased 66 to 75%. At 60 days, the required holding time for cheese made from raw milk, the counts on S-110 medium had

TABLE 2

Organisms enumerated on plate count agar and Staphylococcus Medium No. 110 in samples of Colby Cheese examined at 15-day intervals for 4 mo.[a]

	Age of cheese in days							
	1	15	30	45	60	75	90	120
	GROUP A. Log. average of counts in seven vats of cheese made from good milk inoculated with *Staphylococcus aureus*							
S-110	1,400,000	380,000	350,000	74,000	49,000	55,000	42,000	10,000
PCA	10,000,000	9,100,000	13,000,000	28,000,000	42,000,000	35,000,000	45,000,000	44,000,000
pH	5.40	5.20	5.15	5.15	5.50	5.60	5.45	5.52
	GROUP B. Log. average of counts in four vats of cheese made from mastitic milk (not inoculated)							
S-110	2,800,000	930,000	510,000	470,000	450,000	450,000	35,000	48,000
PCA	2,100,000	20,000,000	10,000,000	11,000,000	14,000,000	21,000,000	8,300,000	4,700,000
pH	5.50	5.31	5.37	5.13	5.21	5.31	5.39	5.39
	GROUP C. Counts in one vat of cheese made from milk aged 16 hr. at 25° C. and inoculated with *S. aureus*							
S-110	51,000,000	13,000,000	12,000,000	16,000,000	19,000,000	5,400,000	120,000	36,000
PCA	720,000,000	260,000,000	220,000,000	270,000,000	180,000,000	170,000,000	100,000,000	60,000,000
pH	5.35	5.35	5.1	5.15	5.35	5.30	5.25	5.55
	GROUP D. Counts in one vat of cheese made from milk aged three days at 1° C. and inoculated with *S. aureus*							
S-110	16,000,000	5,400,000	6,400,000	5,400,000	720,000	93,000	16,000	45,000
PCA	990,000,000	440,000,000	500,000,000	950,000,000	560,000,000	130,000,000	50,000,000	67,000,000
pH	5.55	5.45	5.40	5.25	5.75	5.45	5.40	5.55

[a] Counts per gram.

decreased to 3, 16, 37, and 5% of the populations enumerated on the first day in Groups A, B, C, and D, respectively. These decreases seem logical, since the minimum temperature for growth of *S. aureus* is approximately 10° C. The counts at 120 days were only 0.07 to 1.7% of the counts at one day, with the highest percentage of the surviving staphylococci occurring in the cheese made from the mastitic milk. These results, together with the data in Table 1 which show that during the cheese manufacturing procedure the greatest increase in the population enumerated on S-110 medium occurred in the cheese made from noninoculated milk, suggest that the staphylococci naturally present in the milk were better adapted to the environment of the cheese-making and curing process than the laboratory cultures which were inoculated into the milk.

Acid development was normal in all of the vats during the manufacturing procedure and the pH decreased normally while the cheese was on the press. During the aging period the pH varied from 5.1 to 5.75, with a progressive decrease prevailing during the first 30 to 45 days, after which there was a gradual increase (Table 2). There was no evident correlation between pH of the cheese and the counts on either medium, or between pH and the percentage of coagulase-positive organisms appearing on S-110 medium.

Incidence of coagulase-positive staphylococci in cheese held at 10° C. for 120 days. The data in Table 3 show the results of the coagulase tests on colonies picked from S-110 medium at each interval when the cheese was plated. As the age of the cheese increased from one to 120 days the total percentage of coagulase-positive colonies recovered at each examination interval decreased from 84 to 28%, but the decrease was not continuously uniform, as indicated by comparing the percentages obtained at 45, 60, 75, and 90 days. Minor fluctuations in the general trend, as well as variations in the individual groups of cheese, may be attributed to inherent errors in sampling. Coagulase-positive staphylococci were regularly obtained from cheese stored for 75, 90, and 120 days at 10° C., even when the cheese was made from noninoculated milk. A comparison of the data from the cheese made from inoculated milk (Groups A, C, and D), and the cheese made from noninoculated mastitic milk (Group B), shows that the cheese made from mastitic milk generally yielded smaller percentages of coagulase-positive colonies, based upon the total number of colonies tested, than the cheese made from inoculated milk. For the entire 120-day holding period the percentage of coagulase-positive colonies in each group (Table 3) varied from 35 to 75%, with the lowest percentage occurring in the cheese made from the noninoculated milk. These results are logical, since only a portion of the random contamination of staphylococci contributed by the cow and the environment would be coagulase-positive; whereas, the milks used to make the cheese in Groups A, C, and D were inoculated with a coagulase-positive strain of *S. aureus*. Mattick *et al.* (12) were unable to recover coagulase-positive staphylococci from Cheddar Cheese after it had been aged 14 to 22 wk. Hendricks *et al.* (9) reported that coagulase-positive, β-hemolytic *S. aureus* were found in 75 out of 84 samples of natural American Cheddar Cheese which was from 4 to 8 mo. old.

TABLE 3

Results of coagulase tests performed on colonies picked from Staphylococcus Medium 110

	Age of cheese in days								Coagulase-positive colonies in each group during entire storage period
	1	15	30	45	60	75	90	120	
GROUP A. Seven vats of cheese made from good milk inoculated with *Staphylococcus aureus*									
Number of colonies examined	33	35	20	27	39	41	36	41	
Number of coagulase-positive colonies	28	24	19	13	24	18	15	14	57%
GROUP B. Four vats of cheese made from mastitic milk (not inoculated)									
Number of colonies examined	24	24	23	23	24	19	22	24	
Number of coagulase-positive colonies	18	20	5	2	0	1	5	3	35%
GROUP C. One vat of cheese made with milk aged 16 hr. at 25° C. and inoculated with *S. aureus*									
Number of colonies examined	6	6	6	6	6	6	6	6	
Number of coagulase-positive colonies	6	6	3	2	0	4	4	6	65%
GROUP D. One vat of cheese made with milk aged three days at 1° C. and inoculated with *S. aureus*									
Number of colonies examined	6	6	6	6	4	6	6	11	
Number of coagulase-positive colonies	6	6	6	5	4	6	5	0	75%
Coagulase-positive colonies at each analysis interval	84%	79%	60%	35%	38%	40%	41%	28%	

Previous workers (1, 17) have suggested that minimum populations of 500,000 coagulase-positive staphylococci per gram are required to produce sufficient toxin to cause food poisoning and that foods incriminated with outbreaks often contain millions of staphylococci. However, the ability of staphylococci to produce enough enterotoxin to cause food poisoning may be intimately related to environmental circumstances, which in turn may vary with different foods.

In the work reported herein, two lots of cheese (Groups C and D) were made from milks aged to simulate poor quality and inoculated with *S. aureus*. The total counts in these milks, immediately prior to the addition of the starter, were 10,000,000 and 630,000 per milliliter in Groups C and D, respectively (Table 1). The data in Table 2 show that the organisms capable of growing on S-110 medium apparently were not inhibited and, during the first 60 days of storage, maintained a higher population in this cheese, than in the cheese made from low-count milk. It is also noteworthy that the two lots of cheese made from high-count milk yielded a higher percentage of coagulase-positive staphylococci than the cheese made from the milks with the lower total populations (Table 3). However, this phenomenon may be more intimately related to the characteristic of the strain of *S. aureus* than to the mass population in the milk.

During the progress of this work, 276 colonies which gave coagulase-negative results were picked from cheese samples plated on S-110 medium. These organisms were identified for morphology and Gram reaction. Two hundred thirty-two, or 84%, were Gram-positive cocci, 33, or 12%, were Gram-positive rods, two, or 0.7%, were yeasts, and the remaining nine, or 3.3%, were not characterized.

Coagulase reaction of enriched cheese–sodium citrate blends. Wilson *et al.* (17) applied a rapid test involving agitation and preincubation, and obtained coagulase-positive reactions on custard and ham substrates containing as few as 2,200 and 29,000 staphylococci per gram, respectively. This test is intended to measure the ability of *S. aureus* to produce a measurable amount of coagulase in a food product, when subjected to nutritional enrichment and favorable incubation temperature. Therefore, one would expect to find the greatest incidence of positive tests associated with the highest populations of organisms growing on S-110 medium. However, this was not the case when a modification of the test was applied to the cheese–sodium citrate blends prepared from the cheese used in this work (Table 4). Only 15% of the samples tested were positive and the samples giving coagulase-positive results were not particularly identified with the samples containing high counts on S-110 medium. Populations enumerated on S-110 medium varied from 14,000 to 12,000,000 per gram of cheese in cheese–sodium citrate blends which gave a coagulase-positive reaction. The pH of these blends ranged from 5.45 to 5.80. Coagulase-negative tests were obtained on cheese blends containing from 120 to 51,000,000 organisms per gram of cheese when enumerated on S-110 medium. In several instances where a positive coagulase test was obtained with the cheese–sodium citrate blend no coagulase-positive colonies were found among those picked from the plates of

TABLE 4

Results of coagulase tests performed on cheese–sodium citrate blends

Vat No.	Age of cheese in days							
	1	15	30	45	60	75	90	120
	GROUP A. Cheese made from good milk inoculated with *Staphylococcus aureus*							
1	*	*	*	−	+	−	−	−
2	*	−	+	*	−	−	−	−
3	+	−	+	*	−	−	−	−
4	−	−	*	−	−	−	−	−
5	−	+	*	−	−	−	−	−
6	−	+	+	+	+	−	−	−
7	−	−	−	−	−	−	−	−
	GROUP B. Cheese made from mastitic milk (not inoculated)							
8	−	−	−	−	−	−	−	−
9	+	−	−	−	−	−	−	−
10	−	−	−	+	−	−	−	−
11	−	−	−	−	−	−	−	−
	GROUP C. Cheese made from milk aged 16 hr. at 25° C. and inoculated with *S. aureus*							
12	−	−	+	−	−	+		−
	GROUP D. Cheese made from milk aged three days at 1° C. and inoculated with *S. aureus*							
13	−	−	−	+	−	−	−	−

* Not determined.

S-110 medium. Also, several of the cheese–broth cultures giving negative coagulase tests were subcultured into fresh brain heart infusion–mannitol salt broth and incubated. Coagulase tests on these subcultures were always positive.

The results reported herein do not show any consistent correlation between the coagulase reaction of the enriched blend and the number or coagulase reaction of the organisms appearing when the cheese is plated on S-110 medium. Negative tests on blends containing high populations of coagulase-positive *S. aureus* and positive tests on blends containing few *S. aureus* may be related to environmental circumstances influencing coagulase production.

Reports in the literature (7, 9, 11, 13, 14) indicate that cheese has been responsible for many cases of staphylococcal food poisoning. Previous workers (1, 17) have found populations of 500,000 coagulase-positive staphylococci per gram in food containing clinical levels of enterotoxin. In view of these results, it seems reasonable to suggest that until we have a reliable test for enterotoxin from staphylococci, cheese which attained a population of several hundred thousand coagulase-positive staphylococci per gram during production or ripening should be regarded as a potential source of food poisoning. The work reported herein shows that maximum populations enumerable on S-110 medium (staphylococci) are apt to be present when the cheese is one day old, or shortly thereafter, and microbiological analyses should be made at this time. Subsequent reductions in population which may occur during ripening for 60 days or more may not make the cheese less dangerous.

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FATTY ACID COMPOSITION OF LIPIDS EXTRACTED FROM RATS FED MILK FAT, CORN OIL, AND LARD[1]

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SUMMARY

The lipids extracted from rats fed milk fat differed in both minor and major components from the lipids extracted from rats fed corn oil or lard. The minor components consisted of 1.7–5.4% odd- and branched-chain fatty acids which were found in milk fat and in the lipids extracted from the carcass of rats fed milk fat, but not in corn oil or lard or in the lipids extracted from rats fed corn oil or lard.

The major component fatty acids in milk fat, corn oil, and lard were selectively deposited in the carcass and liver tissue. The most drastic selectivity involved linoleic acid, which varied from 2% in those fed milk fat to 39% in those fed corn oil. On the other hand, the lipids extracted from the liver of rats fed milk fat contained 3% more arachidonic acid than the lipids extracted from livers of those on corn oil, or 13 and 10%, respectively. Heated milk fat in the diet did not seem to alter statistically the mixed fatty acid composition of the carcass or liver lipids.

Previous studies have shown that the composition of body fat and liver lipid is significantly affected by changes in the characteristics of the dietary fat (2, 3, 4, 6, 9, 10, 12, 15, 18, 19). The liver plays an important role in the intermediary metabolism of lipids, modifying them for use in other tissues (5). As thermal oxidation seems to influence the mixed fatty acid composition of a fat (14), the ingestion of excessively heated fats may affect the normal functioning of liver and alter body fat composition. In the present study, fresh and thermally oxidized milk fat, fresh corn oil, and fresh lard were fed to weanling rats and the effect on the mixed fatty acid composition of the carcass and liver lipids was determined.

EXPERIMENTAL PROCEDURE

Preparation of fresh and thermally oxidized milk fat. Butter was melted in a hot water bath and the fat was extracted with ethyl ether in a separatory funnel. The ether extract was washed three times with distilled water and dehydrated over anhydrous sodium sulfate. The ether was removed under vacuum at 40° C., using a Rinco Rotatory evaporator. The fat was stored in the refrigerator at 0° C.

The thermal oxidation was carried out in a three-liter round-bottom flask heated with a heating mantle. The temperature was maintained at 200 ± 1° C. One thousand grams of fresh milk fat was poured into the flask and was heated to about 200° C. under constant stirring with a mechanical stirrer. Air was bubbled through the fat at the rate of 100 ml. per minute with the aid of a

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fritted glass filter stick. Milk fat samples were heated for various periods; namely, 4, 8, 18, and 24 hr. The flask was disconnected from the system after heating for the desired length of time and rapidly cooled to room temperature. The fat was then stored in the refrigerator until used for the feeding experiments.

Growth studies. Male weanling albino rats were divided into groups of six animals each and fed ad libitum a diet which contained 44% cerelose, 31% casein, 4% Wesson Salts (14), and 20% of the test fat. Linoleic acid content of the diets containing fresh and thermally oxidized milk fat was equalized by adding methyl linoleate. The diet was supplemented with 2 g. of a water-soluble vitamin mixture per kilogram of diet. The vitamin mixture consisted of choline chloride 93.5 mg., thiamine hydrochloride 1.24 mg., riboflavin 1.24 mg., pyridoxine 1.24 mg., calcium panthothenate 2.48 mg., folic acid 0.30 mg., and 1.9 g. of glucose. One drop per rat of the following fat-soluble vitamin mixture was administered once each week: Vitamin A (200,000 U.S.P. units) 5 g., vitamin E (mixed tocopherols) 2.535 g., and vitamin D_2 0.0054 g. in 100 ml. of olive oil.

Extraction of fat from carcass and liver. At the end of 4 wk. the rats were fasted for 24 hr., killed by heart puncture, the livers removed, weighed, and minced with sodium sulfate. The livers were extracted three times with an ethanol: ether mixture (1:1) and the solvent was removed under vacuum. The lipid residue was extracted with ethyl ether, dehydrated with sodium sulfate, and the solvent removed under vacuum. The lipid residue was then used directly for methanalysis.

The carcass was transferred to a 2-liter beaker, 100 ml. of 20% hydrochloric acid added, and the beaker heated on a steam bath for 24 hr. The mixture was cooled, transferred to a separatory funnel, and the fat extracted three times with Skellysolve F. The combined extracts were washed with water until free from acid, dehydrated with anhydrous sodium sulfate, filtered, and the solvent removed under vacuum. The lipid residue was stored in the refrigerator at 0° C.

Preparation of methyl esters. Three grams of the lipid were dissolved in 20 ml. ethyl ether in a 100-ml. round-bottom flask, and 20 ml. of sodium methoxide solution added. The sodium methoxide solution was prepared by dissolving 0.5 g. of sodium in 200 ml. absolute methyl alcohol. The flask was left at room temperature overnight and the methyl esters recovered by diluting the reaction mixture with about five volumes of water, acidifying with dilute hydrochloric acid, and extracting the esters with ethyl ether. The ether extract was washed with water, dehydrated over anhydrous sodium sulfate, and the solvent removed under vacuum. The methyl esters were used directly for gas chromatographic analysis. In the case of liver lipid, smaller amounts of the reagents were used, the amount depending upon the weight of fat available.

Gas-liquid chromatography of the methyl esters. The methyl esters were analyzed by gas-liquid chromatography on a 5-ft. Butanediol-succinate (Craig) column at 220° C. with a helium flow rate of 60 ml. per minute. Methyl esters of pure fatty acids were used as references. These included methyl esters of saturated fatty acids (from C_4 to C_{20} straight-chain fatty acids), methyl pal-

TABLE 1

Component fatty acids of body fats extracted from rats fed fresh milk fat, heated milk fat, fresh corn oil, and fresh lard

Fatty acids	Milk fats heated for					Corn oil	Lard
	0	4	8	18	24		
	(hr.)						
Caproic	0.2 ± 0.04[a]	0.2 ± 0.32	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.04	0.0	0.0
Caprylic	0.3 ± 0.17	0.3 ± 0.17	0.3 ± 0.11	0.2 ± 0.08	0.2 ± 0.02	0.0	0.0
Capric	0.8 ± 0.32	0.9 ± 0.22	1.1 ± 0.13	0.9 ± 0.06	1.0 ± 0.43	0.1 ± 0.01	0.1 ± 0.05
C_{11}	0.2 ± 0.19	0.2 ± 0.02	0.2 ± 0.04	0.2 ± 0.05	0.2 ± 0.03	0.0	....
Lauric	1.9 ± 0.65	2.1 ± 0.78	2.3 ± 0.79	2.6 ± 0.39	2.1 ± 0.07	0.3 ± 0.13	0.4 ± 0.07
C_{13}	0.1 ± 0.06	0.1 ± 0.08	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.01	....	....
$C_{13.7}$	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	....	....
Myristic	7.5 ± 0.52	8.2 ± 0.14	8.3 ± 0.69	8.9 ± 0.88	8.5 ± 0.81	1.3 ± 0.13	1.8 ± 0.25
Tetradecenoic	1.2 ± 0.29	1.3 ± 0.32	1.8 ± 0.30	1.6 ± 0.23	1.6 ± 0.38	0.1 ± 0.02	0.1 ± 0.01
C_{15}	0.6 ± 0.36	0.9 ± 0.24	1.4 ± 0.18	1.4 ± 0.34	1.4 ± 0.37	0.1 ± 0.04	0.1 ± 0.03
$C_{15.6}$	0.2 ± 0.04	0.2 ± 0.03	0.2 ± 0.06	0.2 ± 0.05	0.2 ± 0.04	....	....
Palmitic	27.6 ± 1.04	25.0 ± 0.74	24.9 ± 3.15	24.1 ± 1.58	25.7 ± 1.82	19.0 ± 0.75	24.3 ± 1.09
Hexadecenoic	8.3 ± 1.85	9.9 ± 0.39	10.4 ± 0.95	11.0 ± 0.88	10.5 ± 1.17	4.5 ± 0.46	4.4 ± 0.21
C_{17}	0.2 ± 0.05	0.6 ± 0.29	0.5 ± 0.09	0.6 ± 0.09	0.9 ± 0.19	....	....
$C_{17.6}$	0.3 ± 0.16	0.9 ± 0.41	0.9 ± 0.13	1.5 ± 0.62	1.8 ± 0.58	....	....
Stearic	4.4 ± 0.26	3.4 ± 0.55	4.0 ± 1.17	4.2 ± 0.69	3.8 ± 0.54	2.8 ± 0.21	4.9 ± 0.11
Oleic	44.0 ± 1.38	43.1 ± 0.75	40.4 ± 4.54	39.0 ± 2.31	38.3 ± 2.18	31.8 ± 0.88	44.5 ± 0.76
Linoleic	2.1 ± 1.22	2.0 ± 0.36	2.7 ± 0.87	2.7 ± 0.55	3.8 ± 0.57	39.1 ± 1.27	18.5 ± 0.77
Linolenic	0.0	0.0	0.0	0.0	0.0	0.5 ± 0.04	0.5 ± 0.05
Arachidic	0.2 ± 0.05	0.2 ± 0.05	0.3 ± 0.03	0.2 ± 0.04	0.2 ± 0.02	0.3 ± 0.06	0.4 ± 0.05

[a] Average of six samples in each group with their standard deviations.

.... Indicates too small an amount to be detectable.

mitoleate, methyl oleate, methyl linoleate, methyl linolenate, and methyl arachidonate. Each of these reference methyl esters was gas-chromatographed. The retention times of the saturated methyl esters were plotted against chain length on semilog paper. The various fatty acids were identified from the retention times of their methyl esters. The branched-chain fatty acids were tentatively identified from their carbon number as suggested by Woodford *et al.* (22). The fatty acid composition of the gas-chromatographed sample was determined by calculating with the aid of a planimeter the area under the peaks.

RESULTS

The results obtained on gas-liquid chromatography of the methyl esters indicated that the lipids extracted from rats fed milk fat differed in both minor and major component fatty acids from the lipids extracted from rats fed corn oil or lard (Table 1). In addition to 0.2% caproic and 0.3% caprylic acids, the minor components in the carcass lipids from rats fed milk fat consisted of a total of 1.7% fatty acids tentatively identified as odd- and branched-chain (C11-C17) fatty acids. The liver lipids contained only 0.5% of odd-chain and no branched-chain fatty acids (Table 3). These fatty acids were present in milk fat at a total concentration of 5.4%, but were not present in corn oil or lard, nor in the lipids extracted from rats fed corn oil or lard (Table 1). The fatty acid composition of milk fat agreed fairly closely with that reported by Patton *et al.* (13).

Milk fat contained a total of 2.2% branched-chain and the carcass lipid of rats fed milk fat contained 0.6% of these fatty acids. The methyl esters of the branched-chain acids were tentatively identified as 12-methyl tridecanoate, 14-methylpentadecanoate, and 16-methylheptadecanoate. The identification was

TABLE 2

Component fatty acids of rats fed three fats

Fatty acids	Milk fat	Corn oil	Lard
Butyric	4.7	.....	.....
Caproic	2.8	.....	.....
Caprylic	2.1	.....	.....
Capric	3.3	.....	.....
C_{11}	1.0	.....	.....
Lauric	3.7	.....	.....
Dodecenoic	0.2	.....	.....
C_{13}	0.2	.....	.....
$C_{13:7}$	0.4	.....	.....
Myristic	9.8	.....	1.8
Tetradecenoic	1.8	.....	.....
C_{15}	1.3	.....	.....
$C_{15:6}$	1.1	.....	.....
Palmitic	30.4	13.8	28.2
Hexadecenoic	1.9	.....	2.6
C_{17}	0.7	.....	.....
$C_{17:6}$	0.7	.....	.....
Stearic	10.6	2.1	16.5
Oleic	22.3	29.8	41.7
Linoleic	1.2	54.5	9.3
Arachidic	0.2		

TABLE 3

Component fatty acids of liver lipids extracted from rats fed fresh milk fat, heated milk fat, fresh corn oil, and fresh lard

Fatty acids	Milk fats heated for					Corn oil	Lard
	0	4	8	18	24		
	(hr.)						
Capric	0.3 ±0.03[a]	0.3 ± 0.09	0.3 ± 0.13	0.2 ± 0.01	....	....	....
Lauric	0.7 ± 0.13	0.4 ± 0.08	0.3 ± 0.04	0.2 ± 0.04	....	....	....
Myristic	1.8 ± 0.21	1.6 ± 0.31	1.5 ± 0.64	0.6 ± 0.05	0.6 ± 0.10	1.1 ± 0.13	0.7 ± 0.07
C_{15}	0.2 ± 0.06	0.2 ± 0.02	0.3 ± 0.06	0.2 ± 0.02	0.3 ± 0.06	....	....
Palmitic	24.9 ± 0.93	27.1 ± 1.75	30.4 ± 1.71	32.3 ± 1.61	34.5 ± 0.45	21.0 ± 1.16	24.0 ± 0.44
Hexadecenoic	4.2 ± 0.29	4.1 ± 0.83	4.6 ± 0.74	3.6 ± 6.79	3.8 ± 0.21	1.1 ± 0.30	1.2 ± 0.35
C_{17}	0.3 ± 0.03	0.4 ± 0.08	0.4 ± 0.12	0.4 ± 0.03	0.6 ± 0.01	....	....
$C_{17:1}$	0.3 ± 0.03	0.4 ± 0.06	0.4 ± 0.11	0.4 ± 0.05	0.6 ± 0.03	....	....
Stearic	22.2 ± 1.09	20.9 ± 0.78	19.2 ± 2.05	23.8 ± 1.22	22.5 ± 0.99	11.8 ± 0.12	20.7 ± 2.04
Oleic	28.0 ± 1.56	27.1 ± 2.88	27.1 ± 0.86	25.6 ± 0.98	24.5 ± 2.14	17.3 ± 0.79	27.9 ± 1.08
Linoleic	4.1 ± 0.50	4.1 ± 0.32	4.1 ± 0.48	3.6 ± 0.21	3.8 ± 0.13	37.5 ± 1.18	14.1 ± 0.80
Arachidonic	13.4 ± 0.71	13.8 ± 0.56	11.3 ± 0.65	9.4 ± 0.34	9.5 ± 1.17	10.2 ± 0.32	11.4 ± 0.97

[a] Average of three liver samples in each group with their standard deviations.
.... Indicates too small an amount to be detectable.

based on their carbon numbers, which agreed with those reported by Woodford *et al.* (22) and the fact that the branched-chain esters are eluted before the corresponding straight-chain esters on polar phase.

The major component fatty acids in milk fat, corn oil, and lard were reflected in the carcass and liver tissue fat. The most drastic change involved linoleic acid, which varied from 2% in those fed milk fat to 39% in those fed corn oil. As with linoleic acid, the hexadecenoic or (palmitoleic) and oleic acid content of the carcass and liver lipids were selectively increased in comparison to their concentration in milk fat. The carcass lipids of animals fed milk fat contained 2.1, 8.3, and 44.0%, and the liver lipids 4.1, 4.2, and 28.0% linoleic, palmitoleic, and oleic acid. In comparison, milk fat contained only 1.2, 1.9, and 22.3% of these respective fatty acids. The oleic acid content of the lipids of animals fed corn oil or lard did not differ substantially from the oleic acid content of the dietary fats. However, the linoleic acid content of the lipids obtained from animals fed corn oil was significantly less than the linoleic acid content of corn oil, or 39.1 and 54.5%, respectively.

The arachidonic acid content of the liver lipids obtained from animals fed milk fat was significantly greater than for those fed corn oil, in spite of the low dietary linoleic and arachidonic acid level of milk fat. The liver lipids of those fed milk fat contained 13.4% and those on corn oil or lard contained 10.2 and 11.4% arachidonic acid, respectively. The high arachidonic acid level of the liver lipids was accompanied by a higher stearic acid level than the carcass lipids possessed, or 22.2, 11.8, and 20.7% stearic acid for liver lipids, as compared with 4.4, 2.8, and 4.9% stearic acid for carcass lipids, respectively. The lauric, myristic, and palmitic acid content of the lipids extracted from animals fed milk fat did not differ substantially from those fed corn oil or lard. The palmitic acid level of those fed milk fat was somewhat higher than those fed corn oil or lard, or 27.6, 19.0, and 24.3%, respectively.

The carcass and liver lipids of animals fed heated milk fat did not contain hydroxy acids. The carcass lipids of rats fed milk fat heated for 24 hr. contained 1.5% more C_{18} branched chain and 6% less oleic acids, and the liver lipids contained 9.6% more palmitic and 4.9% less arachidonic acid than those fed fresh milk fat. These changes in percentage composition may have been a result of difference in weight gain and food intake (Table 4). Rats fed fresh milk fat gained an average of 147 g. and those fed milk fat heated for 24 hr. gained 109 g. in 4 wk. However, when growth was compared on an equalized food intake, no statistical difference in growth was observed. Although a larger liver to body weight ratio was noted in rats fed heated fat, no abnormality in appearance of the liver was noted.

DISCUSSION

The present results confirm those obtained in previous studies (2, 3, 9, 12, 19), which indicated that a particular fatty acid can be selectively deposited in animal tissue. Gas liquid chromatography of the fatty acid methyl esters revealed the presence of a number of acids previously overlooked in the depot fat of rats fed milk fat. Short-chain fatty acids C_6, C_8, and C_{10} were identified

TABLE 4

Effect of feeding corn oil, lard, and fresh and thermally oxidized [a] milk fat on the growth of rats

Fat used	Average gain in weight of rats (4 wk.)	Average food intake (4 wk.)	Gain in weight per 100 g. food intake	Per cent liver/body weight	Average weight carcass fat *(g.)*	Average weight liver fat *(mg.)*
Fresh milk fat	147.0 ± 6.71 [b]	292.8	50.2 ± 3.06	4.6 ± 0.74	22.1 ± 1.41	372.3 ± 36.1
Milk fat heated 4 hr.	137.0 ± 4.04	280.3	48.9 ± 1.99	5.3 ± 0.25	19.1 ± 1.88	374.0 ± 17.9
Milk fat heated 8 hr.	122.8 ± 8.64	257.8	47.7 ± 1.96	5.8 ± 0.39	15.9 ± 2.34	358.5 ± 23.3
Milk fat heated 18 hr.	129.5 ± 3.77	273.8	47.4 ± 2.28	5.8 ± 0.42	19.5 ± 1.52	345.3 ± 15.2
Milk fat heated 24 hr.	108.8 ± 6.81	231.0	47.1 ± 2.44	6.8 ± 0.73	12.2 ± 0.75	336.5 ± 21.2
Fresh corn oil	144.2 ± 6.01	286.5	50.3 ± 1.42	4.5 ± 0.19	19.7 ± 1.11	321.0 ± 15.4
Fresh lard	142.0 ± 6.13	286.0	49.6 ± 1.25	4.5 ± 0.19	19.3 ± 0.76	311.5 ± 18.4

[a] Milk fat heated at 200° C. and air bubbled at the rate of 100 ml/min.
[b] ± Standard deviation.

in small amounts when the rats received fresh or thermally oxidized milk fat in their diets. The analyses of depot fats further revealed the presence of fatty acids with odd-numbered carbon atoms such as C_{11}, C_{13}, C_{15}, and C_{17} and the branched-chain fatty acids, namely C_{14}, C_{16}, and C_{18}. Channon *et al.* (3) and Bose *et al.* (2) did not report the presence of the odd-numbered carbon fatty acids and branched-chain fatty acids in their analyses of the depot fats of rats on milk fat diets. Hansen *et al.* (7, 8, 16) have positively identified the presence of odd-numbered carbon fatty acids and the branched-chain fatty acids in ox fat, mutton fat, and milk fat. The work carried out in our laboratory on the analyses of depot fat of rats receiving corn oil or lard has indicated the absence of these fatty acids, indicating thereby that the branched-chain and odd-chain fatty acids are not synthesized by the rat. The odd-chain and branched-chain fatty acids peculiar to milk fat (10) probably have their origin in the microorganisms which are normally found in the intestinal tract of ruminants and are eventually stored in the depot fat of rats when they receive milk fat in their diets. This observation is in agreement with the findings of Livingston *et al.* (11), who showed that 10% of 14-methylhexadecanoic acid supplemented to a fat free diet is deposited in the body fat of the rat.

Palmitic acid is the chief constituent of the saturated fatty acids of depot and liver fats of rats and its formation does not seem to depend upon the dietary palmitic acid. Palmitoleic acid is found in substantial amounts in body fat and is probably formed by the breakdown of oleic acid or by desaturation of palmitic acid. Very little, if any, linoleic acid is synthesized by the rat, as the amount of linoleic acid is found to be entirely dependent on the linoleic acid derived from the diet. When the linoleic acid content of the dietary fat was low, as in the case of milk fat, the tissue contained a slightly higher concentration of linoleic acid than was present in milk fat. However, when the linoleic acid content of the dietary fat was high, as in corn oil, the tissue did contain a large amount of linoleic acid, but the concentration was not as high as was in the dietary fat. A mere comparison of the linoleic acid content of the dietary fat (1, 15, 17, 21), therefore, does not reflect the ultimate concentration or the role of linoleic acid in an animal tissue. With an increase in the linoleic acid in the depot fat, there was a relative decrease in the palmitoleic acid. This is in confirmation of the results reported by Tove *et al.* (18). The similarity of the physical properties of linoleic and palmitoleic acids suggests that there is a tendency on the part of the animal to maintain the desirable properties of body lipids by reducing the synthesis of palmitoleic acid when the animal receives a large amount of dietary linoleic acid. Oleic acid is the most important unsaturated fatty acid found in rat depot fat. The concentration of this acid is, however, not influenced by the dietary oleic acid, as part of it is synthesized from saturated fatty acids, which in turn may be derived from carbohydrate and protein in the food.

The liver plays an important and unique role in the metabolism of lipids. It is believed that the newly absorbed fat is worked over in the liver to alter the nature of its fatty acid pattern so that it will conform to the type usually stored

by the particular tissue. This synthesized fat is then transferred to the fat depots for storage. Although the dietary fat (milk fat) consisted of quite appreciable amounts of lower fatty acids, these were not found in the liver lipid. These results seem to indicate that such fatty acids are deposited in the other tissues before they reach the liver or are oxidized if they are transported to the liver. As in depot fats, the linoleic acid content of liver fat is greatly influenced by dietary linoleic acid. The liver lipids of rats fed milk fat contained a higher concentration of arachidonic acid than those fed corn oil or lard. The ability to convert linoleic acid to arachidonic acid was not lost even under conditions of stress, although somewhat less arachidonic acid was synthesized in rats fed heated as compared to fresh milk fat. Hydrogenation of oleic acid in liver is probably responsible for a significantly large amount of stearic acid in liver lipid.

The presence of heated, instead of fresh, milk fat in the diet did not result in the deposition of hydroxy acids, as found by Perkins *et al.* (14), when heated corn oil was fed. The presence of heated fat seemed to increase the concentration of the fatty acid fraction tentatively identified as a C_{18} branched-chain fatty acid. However, as position identification was not possible, the increase in this fraction could have been due to some other factor.

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INFLUENCE OF FEED UPON THE COMPOSITION OF MILK. II. LOW VERSUS HIGH FAT RATIONS

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SUMMARY

Based on a 12-wk. mild weather switch-back feeding trial involving two groups of three Holstein and two Jersey cows each, this information was obtained. The per cent S.N.F. and the per cent lactose in milk from Holstein cows fed a high fat ration with 6% Marcol B-75 (Ration M) were significantly lower than when fed a control ration (Ration B) which contained 63% more crude fiber and no added fat. No significant differences in composition of milk were observed with the Jersey cows when on Rations M and B.

The per cent fat was significantly higher in milk produced by Holsteins fed a high fat ration with 6% stabilized prime tallow (Ration T) than when fed Ration B. However, the per cent S.N.F. and S.N.F.-lactose were significantly higher on Ration B than on Ration T with this breed. With the Jersey, the only significant difference was a higher per cent fat in milk with Ration T than with Ration B.

Holsteins fed on Ration M produced 75 and 70% of all milk samples below Texas minimum requirements for 3.25% fat and 8% S.N.F., respectively. With Ration B the values were 39 and 43%, and with Ration T, 9 and 61% for fat and S.N.F., respectively.

Based on a 13-wk. mild weather continuous feeding trial involving two groups of five Holstein and five Jersey cows each, the following information was obtained. The per cent fat, S.N.F., and S.N.F.-lactose in milk of Holsteins fed a high fat ration with 6% stabilized prime tallow (Ration T) were significantly lower than for the control group fed the regular herd ration (Ration H) without added fat. However, this was true only for Period II (7th to 13th wk. of trial). With the Jersey group the per cent of the alcohol-soluble portion of milk fat was significantly higher with Ration T than with Ration H in Period I (1st to 7th wk. of trial).

The addition of various fats to rations fed to lactating dairy cows and their effect upon milk yield, per cent fat, and composition of milk fat has been investigated by different workers (1, 4, 6, 9). Whereas Horton *et al.* (6) observed no significant increase in total milk yield and per cent fat in milk during a 33-wk. feeding period of rations containing 4 and 8% added tallow, other workers reported markedly higher total milk yield (1, 9), higher per cent fat in milk (1), and significant differences in the composition of milk fat (9). Both cottonseed oil (1) and cottonseed cake (4) have been reported to increase total milk yield as well as per cent fat in milk.

The first part of this study deals with the influence of rations containing added tallow or a cottonseed oil by-product (Marcol B-75) defined below upon the composition and certain selected properties of milk. The second part deals with the influence of a ration containing added tallow upon the composition of milk and milk fat.

EXPERIMENTAL PROCEDURE

In the first part of this study, 20 lactating dairy cows were equally divided into two lots of six Holsteins and four Jerseys, the two lots being balanced with

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respect to age, body weight, stage of lactation, previous production records, current milk production, and per cent milk fat. Each lot was divided into two groups of three Holsteins and two Jerseys and placed on a feeding schedule as shown in Table 1.

TABLE 1

Feeding schedule during switch-back trial

Four-wk. period	Ration fed to cows of			
	Lot I [a] Group [b]		Lot II [a] Group [b]	
	1	2	1	2
1	B	B	M	T
2	M	T	B	B
3	B	B	M	T

[a] Six Holstein and four Jersey each.

[b] Three Holstein and two Jersey each.

Composition of the concentrate mixtures and the kind and amount of roughage fed in Rations B, M, and T are shown in Table 2.

TABLE 2

Composition of rations fed during switch-back trial

Ingredients	Ration B	Ration M	Ration T
		(lb.)	
Ground ear corn with husk	500	None	None
Corn meal	None	290	290
Ground oats	200	None	None
Ground milo grain	None	300	300
Wheat bran	200	100	100
Cottonseed meal (41% protein)	100	250	250
Modified Marcol B-75 [a]	None	60	None
Stabilized prime tallow	None	None	60
Bone meal	10	10	10
Salt	10	10	10
Alfalfa hay/100 lb. body weight	1.13 [b]	0.75 [b]	0.75 [b]
Atlas silage/100 lb. body weight	2.97 [b]	1.50 [b]	1.50 [b]

[a] A commercial product consisting primarily of the methyl esters of fatty acids as follows: Oleic 23%, linoleic 48%, palmitic 23%, stearic 2%, and others 4% (Marco Chemical Company, Fort Worth, Texas).

[b] Average amount of forage consumed per day.

Control Ration B with no added fat contained 63% more crude fiber than either Ration M or Ration T. The estimated net energy and protein content of the three rations was the same. The rations were fed in such amounts as to furnish 5% more than the estimated net energy requirements of the cows according to the Morrison standard (5).

Individual cows' milk samples taken once a week at the evening milking were examined for certain selected constituents and properties in a manner similar to that described by Peters *et al.* (7).

In the second part of this study two lots of ten cows each—five Holsteins and five Jerseys—were selected as above for a continuous winter feeding trial which lasted 13 wk. Both lots of cows were kept in the same loafing type shelter and pen where they had free access to alfalfa hay, sorghum silage, and water. They received their concentrate part of the ration while stanchioned just prior to milking. The concentrate portion of the control Ration H consisted of 525 lb. ground milo grain, 300 lb. wheat bran, 175 lb. cottonseed meal (41% protein), 10 lb. bonemeal, and 10 lb. salt. The concentrate portion of the experimental Ration T was the same as shown in Table 2. Because the shipment of tallow fed during the first 6 wk. differed somewhat in composition from the shipment fed during the last 7 wk., the feeding experiment was divided into Periods I and II. (See footnote in Table 5.)

Individual cows' milk samples were taken at the evening and morning milking once a week and pooled into four composite samples by breed and ration. A portion of each sample was used for composition tests similar to those made in the first part of this study. The remaining portion of each sample, about 1,700 ml., was centrifuged, the cream churned, and the milk fat of the resulting butter extracted with ether. The dry milk fat was fractionated into alcohol-soluble and alcohol-insoluble portions (2) and the refractive index (R.I.) was determined at 40° C. on the dry fat as well as on the alcohol-soluble portion. Weights of dry fat samples and of its fractions were observed and recorded.

Data obtained were subjected to statistical treatment according to methods described by Brandt (3) and Snedecor (8).

RESULTS

The data in Table 3 show results of the switch-back trials with statistical treatment as indicated. For the Holstein group, significant differences were observed between B ration mean and the mean of Ration M for per cent S.N.F. and per cent lactose, both in favor of Ration B. For the same breed, B Ration mean minus the mean of Ration T resulted in a significant difference in per cent milk fat in favor of Ration T, and in per cent S.N.F. and per cent S.N.F.-lactose both in favor of Ration B.

TABLE 3

Comparison by breed of Ration B with Rations M and T (B ration mean minus the mean of Rations M or T)

Criteria studied	Holstein		Jersey	
	B-M	B-T	B-M	B-T
Conductivity at 37° C.	−10.17 ± 11.7[a]	3.55 ± 7.10	9.04 ± 2.74	7.39 ± 10.1
Conductivity at 0° C.	−2.23 ± 5.25	−3.36 ± 3.64	7.01 ± 4.72	0.13 ± 6.49
Freezing point	0.022 ± 0.019	−0.004 ± 0.042	0.025 ± 0.009	0.051 ± 0.045
Total solids	3.60 ± 1.51	−1.37 ± 1.45	1.69 ± 3.53	−4.53 ± 1.97
Fat	1.73 ± 1.99	−4.48* ± 1.43	−2.07 ± 2.53	−8.55* ± 1.48
Solids-not-fat	1.89* ± 0.50	2.17* ± 0.27	3.96 ± 1.44	4.38 ± 1.93
Lactose	1.25* ± 0.21	−0.13 ± 0.33	−0.05 ± 0.48	−0.35 ± 0.48
S.N.F.-lactose	0.54 ± 0.42	1.95* ± 0.35	3.65 ± 2.04	3.73 ± 1.33

* Significant at 5% level.

[a] Standard error of mean difference.

With the two Jersey groups, the only significant difference observed was with respect to per cent milk fat in favor of Ration T.

The data in Table 4 show the per cent of all milk samples which fell below Texas (State) requirements, arranged by breed and ration. Milk of both breeds produced on different rations was similar in meeting the minimum requirements for freezing point. For the Holstein group, the per cent milk samples below minimum State requirements for Rations B, M, and T was 39, 75, and 9 fat, and 43, 70, and 61 for S.N.F. For the Jersey groups all milk samples met the minimum State requirements for per cent fat and nearly all met requirements for per cent S.N.F.

TABLE 4

Per cent of samples below state requirements by breed and ration

Constituent or property	Minimum requirement for Texas	Holstein			Jersey		
		B	M	T	B	M	T
		(%)					
F. P. *(−° C.)*	0.5335[a]	4	0	3	5	0	4
Fat *(%)*	3.25	39	75	9	0	0	0
S.N.F. *(%)*	8.00	43	70	61	1	0	3

[a] Tolerance of 3% added water.

Data of the continuous feeding trial are shown in Table 5. Although not statistically significant, mean values for S.N.F., lactose, and S.N.F.-lactose were lower for Holsteins in Period I when on Ration T than when on Ration H. Results in Period II showed a similar trend, except that the decrease in mean values with Ration T reached statistical significance in the case of S.N.F., S.N.F.-lactose, and per cent fat in milk.

With the Jerseys, the mean values for per cent fat in milk were higher, although not significantly so, when on Ration T during Period I but not during Period II. The mean value for the alcohol-soluble portion of milk fat was sufficiently higher with Ration T in Period I to be of statistical significance. Although the value remained the same, it was not significant in Period II, due to the rise of the value from Ration H. The mean S.N.F. values were somewhat lower with Ration T than with Ration H in both periods. This was due mainly to a lower S.N.F.-lactose content of milk from Ration T.

DISCUSSION

The feeding of rations containing concentrates B, M, or T to lactating dairy cows had no significant effect upon the properties and composition of milk with respect to its conductivity, freezing point, and per cent T.S. However, Rations M and T, as compared with Ration B, did alter significantly the composition of milk of one or more of the groups with respect to per cent fat, S.N.F., lactose, and S.N.F.-lactose. The higher per cent fat in milk produced by cows of both breeds when on Ration T than when on Ration M suggests a different utilization of the two types of added fat. This is in agreement with results by other workers

TABLE 5

Average response by breed to Ration H and T

Criteria studied	Mean value and standard error of the mean by breed and ration			
	Holstein		Jersey	
	H	T [a]	H	T [a]
Period I				
Fat *(%)*	3.34 ± 0.19	3.40 ± 0.21	5.37 ± 0.19	5.61 ± 0.20
S.N.F. *(%)*	8.56 ± 0.07	8.07 ± 0.23	9.31 ± 0.23	9.13 ± 0.42
Lactose *(%)*	4.96 ± 0.12	4.64 ± 0.11	4.75 ± 0.25	4.76 ± 0.15
S.N.F.-lactose *(%)*	3.60 ± 0.21	3.43 ± 0.29	4.56 ± 0.11	4.37 ± 0.15
R. I. of dry fat at 40° C.	1.4444 ± 0.0029	1.4444 ± 0.0015	1.4448 ± 0.0022	1.4437 ± 0.0027
Alcohol-soluble portion *(%)*	71.1 ± 3.8	64.2 ± 6.1	67.0 ± 8.4	83.8* ± 2.7
R. I. of alcohol-soluble portion at 40° C.	1.4502 ± 0.0009	1.4496 ± 0.0019	1.4478 ± 0.0023	1.4446 ± 0.0016
Period II				
Fat *(%)*	3.80 ± 0.17	3.38* ± 0.25	6.00 ± 0.33	5.88 ± 0.22
S.N.F. *(%)*	8.58 ± 0.17	8.00* ± 0.15	9.11 ± 0.21	9.03 ± 0.17
Lactose *(%)*	4.83 ± 0.15	4.66 ± 0.14	4.63 ± 0.20	4.79 ± 0.10
S.N.F.-lactose *(%)*	3.75 ± 0.15	3.34* ± 0.20	4.48 ± 0.25	4.24 ± 0.20
R. I. of dry fat at 40° C.	1.4446 ± 0.0022	1.4432 ± 0.0033	1.4445 ± 0.0019	1.4463 ± 0.0033
Alcohol-soluble portion *(%)*	66.3 ± 12.0	67.7 ± 6.0	78.6 ± 6.9	84.0 ± 4.2
R. I. of alcohol-soluble portion at 40° C.	1.4487 ± 0.0013	1.4507 ± 0.0019	1.4503 ± 0.0009	1.4498 ± 0.0013

* Significant at 5% level.

[a] Composition of tallow: Period I: R.I. of dry fat 1.4474; alcohol-soluble portion 54.8%; R.I. of alcohol-soluble portion 1.4494.

Period II: R.I. of dry fat 1.4538; alcohol-soluble portion 41.9%; R.I. of alcohol-soluble portion 1.4580.

(10). That different added dietary fats and oils may increase or decrease the per cent fat in milk has also been reported (1).

While it is of interest to note the different effects of the several rations upon the composition of milk on cows of the same breed, the effect of a single ration upon cows of the two breeds is significant. If such observed differences in the utilization of the same ration for the formation of different milk constituents are not due to sampling, they must be atributed to hereditary differences between breeds (7, 10).

Although cows of both breeds produced milk lower in per cent T.S. (by producing lower per cent fat, S.N.F., or both) when on Ration M or T than when on Ration B, such decline in T.S. was accompanied by a 4% higher average daily milk yield (5). The higher daily milk yield would be of benefit to the producer up to the point where the per cent T.S. drops below the legal requirement.

In the continuous feeding trial results with Ration H versus T upon the composition of milk are similar to those of Ration B versus T in the switch-

back trial. The similarity of results is more evident with the Holstein than with the Jersey group. One reason for some of the differences in results may be due to the difference in the feeding regime. During the switch-back trial both the roughage and concentrate intake per cow were rigidly controlled, whereas in the continuous feeding trial only the intake of the concentrate was controlled.

Examination of the data in the lower part of Table 5 indicates that although cows of both breeds are good converters of tallow into milk fat there are, nevertheless, breed differences. Thus, for the alcohol-soluble portions of the milk fat, which showed greater variations than did the R.I. values, the average percentages for Periods 1 and 2 for Ration H versus T was 68.6 versus 67.4 for the Holstein group, and 74.8 versus 82.2 for the Jersey group. The average value for tallow was 48.35%.

On the basis of results obtained in this study, it is evident that the composition of milk is affected by (a) the type of ration, (b) the type of fat added to the ration, and (c) the breed of the animals.

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PRESERVATION AND FEEDING VALUE OF ALFALFA STORED AS HAY, HAYLAGE, AND DIRECT-CUT SILAGE

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SUMMARY

The preservation efficiency and chemical quality of silages made from direct-cut and heavily wilted (haylage) alfalfa were compared in three successive years. Silages were stored in gas-tight steel silos. A minimum of 4% loss of stored dry matter was observed under the haylage system. This was attained by maintenance of the gas-tight condition and provision of a cap of unwilted forage. Very poor preservation of haylage dry matter was obtained when both of these precautions were neglected. Storage losses of high-moisture direct-cut silage were 22-24% of the stored dry matter, although spoilage was a minor problem. Major improvement of silage chemical quality by extended wilting was indicated by statistically significant negative correlations between dry matter content of the stored forage and ammoniacal nitrogen, acetic acid, propionic acid, and total acid. The positive correlation of lactic acid and dry matter was small and not significant and pH was rather constant over the entire range of 20-53% dry matter. High chemical quality of haylage was indicated by low levels of undesirable constituents, although pH was generally high and only small amounts of lactic acid developed.

The feeding value and digestibility of the silages was compared to barn-dried hay made from the same crops. Animal acceptance, milk production, and live weight gains showed barn-dried hay to have, generally, the highest feeding value and direct-cut silage the lowest. Haylage surpassed high-moisture direct-cut silage in these respects with the exception of milk production, which was about equal on the two types of silage.

Dry matter consumption was positively correlated with dry matter content of the silage. High negative correlations were found between silage dry matter consumption and content of volatile organic acids and ammoniacal nitrogen. Lactic acid content and pH showed poor correlations with dry matter consumption. Digestibility coefficients were generally highest for barn-dried hay, lowest for haylage, and intermediate for direct-cut silage.

The improvement of silage quality and preservation by wilting immature hay crops to 65-70% moisture has been reported by many investigators. Reduction of the moisture content below this level has not been generally recommended because of the increased possibility of heating and molding, since it is more difficult to exclude air from the more porous silage mass. Forage containing only 35-45% moisture is successfully ensiled in Italy by the Crema Process (14), in which air exclusion is aided by weighting the silage surface with 600-2,000 lb. per square yard. The advent of gas-tight silos has presented the possibility of storing forage having less than 65-70% moisture but without the inconveniences of the Crema Process. The term haylage has been used to describe this type of silage having a moisture content of about 50%.

Although gas-tight silos had been available for nearly 10 yr. at the start of this experiment, relatively few data were available concerning the effect of crop moisture content on preservation efficiency and silage quality when these structures are used. Shepherd *et al.* (15) compared haylage to wilted silage

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in gas-tight silos. He reported that storage losses of dry matter were about 1% in the haylage and 6% in wilted silage. He noted some difficulty with mold in the haylage. A later report by Voelker (19) has shown no loss from spoilage in haylage and a total weight loss of 2-7%. However, weight loss would tend to be less than dry matter loss in such dry silage, since any water formed by fermentation or respiration would remain in the silage. The possibility of extremely low dry matter losses in relatively dry, well-sealed forages has also been reported by Briggs (1), who used plastic bags, and Langston *et al.* (11), using miniature steel silos.

Most reports showing improvement of silage chemical quality by wilting high-moisture crops are based on wilting to 65-70% moisture, and their applicability to lower moisture levels is not known. Shepherd *et al.* (15) reported that haylage had a higher pH and contained more residual sugar than wilted silage. This was interpreted as indicating a less active fermentation. Woodward and Shepherd (22) had previously reported that a high pH in wilted silage was less objectionable than in high-moisture silage, this concept being supported mainly by animal intake data. This would indicate that the chemical evaluation of silages having a broad range of moisture contents requires considerably more than pH measurements.

Although the feeding value of hay and silage from the same crop has been compared in a number of reports, there appears to be no consistent relationships between the two. This may result from difficulty in controlling and describing quality variations which occur within each class of forage or from differences in the evaluation methods used.

A number of workers have reported dry matter consumption from wilted silage to be lower than that from high-quality hay from the same crop (7, 8, 17, 18). However, Shepherd *et al.* (16) reported somewhat better acceptance of the wilted silage. The digestibility of dry matter in these two forms of the same crop has been reported as lower in the wilted silage (2), about equal (10, 17) and higher in the wilted silage (4). The depression of digestibility by heating of either hay or silage was, presumably, not a factor in these reports. The replacement of hay with wilted silage on an ad libitum basis has, in most cases, resulted in reduced milk production or live weight gains (3, 8, 18). Shepherd *et al.* (16) reported equal production when substitution was made on an equalized dry matter basis. Cornell workers have recently published results from ten comparisons of wilted silage and barn-dried hay for dairy cows on an ad libitum basis (17). The dry matter consumption from silage was consistently below that of the hay. Significantly more milk was produced from silage in two comparisons, but no essential difference in this respect occurred in most of the comparisons.

An improvement of silage feeding value by wilting as compared to direct-cutting has been frequently reported (5, 12, 13, 22). However, this improvement is not invariably found (21). Few investigators have attempted to determine the improvement obtainable by wilting to less than 65% moisture, because this is generally considered to be the lower moisture limit for conventional

silos. Data obtained by Shepherd *et al.* (15) indicated some further reduction in moisture content below 65% would give further improvement in feeding value. This was evidenced largely by greater animal acceptance of the drier material.

The objectives of this experiment were: (1) To determine the relationships of forage moisture content, over a broad range, to silage preservation and chemical quality when stored in gas-tight silos. (2) To determine the relative feeding value and digestibility of these silages as compared to barn-dried hay made from the same crops. These determinations were made under conditions of ad libitum forage feeding and equalized grain supplementation.

EXPERIMENTAL PROCEDURE

Hay, haylage, and direct-cut silage were prepared in each of three successive years: 1957 (Experiment 1), 1958 (Experiment 2), and 1959 (Experiment 3). Harvesting and storage procedures were generally similar each year, the three forms of forage being harvested simultaneously from the same crops. Haylage and direct-cut silage were chopped with forage harvesters to a $\frac{5}{16}$-in. theoretical length and stored in gas-tight silos. In-put weights and samples were obtained from each load of forage, the samples were analyzed for dry matter, sugar, carotene, and proximate analysis, and these values used in calculation of quantitative storage losses. Hay, barn-dried with heated air, was prepared without in-put weights or samples.

Second- and third-cutting alfalfa was used in Experiment 1. Thus the filling of the silos was intermittent with silos closed excepting during the filling periods. First-cutting alfalfa was harvested May 27-June 5 for Experiment 2, and harvested May 22-28 for Experiment 3.

Seepage from the direct-cut silage in Experiments 2 and 3 was measured with a meter and analyzed for dry matter content. This measurement was not complete, however, since some seepage which occurred around the doors and silo foundation was lost.

Weights and samples of silage and haylage were obtained daily during the out-feeding period in each experiment. These samples were analyzed for the following constituents: dry matter, crude protein, ether extract, crude fiber, ash, ammoniacal nitrogen, carotene, total sugars expressed as glucose, butyric, propionic, acetic and lactic acid, and hydrogen ion concentration expressed as pH. The methods employed were those recommended by a recent conference of silage investigators (23). These values, as well as those for input, seepage and spoilage, were the basis for determining the preservation efficiency and chemical quality characteristic of the silages. Daily samples were stored at 35° F. All determinations except proximate analysis were made on five-day composites and proximate analyses were determined on dried 25-day composites.

The separation of spoilage and feedable silage was necessarily on a somewhat arbitrary basis. Material which was noticeably moldy, slimy, or had a strong odor of ammonia was termed spoilage.

A comparison of the production value and digestibility of the two silages and one hay was made with milking cows each year. Experiment 1 (1957) forages were evaluated by a 90-day continuous type feeding trial with four cows assigned to each type of forage. This design was adopted when it became apparent that the supply of good-quality haylage might be insufficient for a more balanced design and a longer feeding period.

Experiment 2 (1958) forages were evaluated in a 120-day feeding trial, using four trios of milking cows. The design consisted of four 3 × 3 Latin squares with 40-day periods. The first ten days of each period was regarded as a change-over period.

The feeding trial for evaluating Experiment 3 (1959) forages was similar to that of Experiment 2, except that five Latin squares were used with three cow trios starting September 3, followed by two trios which started October 13.

Feeding procedures and the data obtained were similar in each experiment. Cows were fed individually twice daily and refused feed weighed back once daily. Rations consisted of the experimental forage to the extent of appetite and a 16% crude protein grain mixture. Initial grain rations were assigned individually on a grain: FCM ratio of about 1:4 in Experiments 1 and 2 and 1:5 in Experiment 3. Subsequent grain adjustments were made each ten days on the basis of the average production decline for all cows on the experiment. FCM production was calculated from daily milk weights and the percentage milk fat which was determined each ten days by the Babcock method. Live-weights were obtained on the last three days of each ten-day period.

Analyses of a part of the samples routinely obtained to determine total out-put of the silos were also utilized in connection with the feeding trials. Hay samples were obtained at five-day intervals by boring the bales to be fed. The proximate analysis of these borings was determined as well as the official grade. The dry matter content of refused feeds was determined each five days and that of the grain fed each 20 days, by oven drying.

Apparent digestibility of dry matter and nutrient constituents of the forages was determined by the chromic oxide-grab sample technique described by Kane *et al.* (9). These determinations were made simultaneously with the feeding trials and the same animals were used for both purposes. Digestibility trials were carried out when the feeding trials were about one half completed.

RESULTS

Values for the average chemical composition of silages when stored and after storage are presented in Table 1. The relatively high initial dry matter content of the direct-cut forage in Experiment 1 is a reflection of the second- and third-cutting crops used in this experiment. Dry matter content of the stored haylage ranged from 39% in Experiment 3 to 50% in Experiment 1. The low level of the former value was caused in part by the inclusion of two loads of direct-cut forage for sealing the haylage.

Some year-to-year differences in initial crops are evident in the proximate analysis values (Table 1), but differential effects of ensiling method on these

values were not marked. All haylages showed a greater retention of sugar but a greater loss of carotene then the comparable direct-cut silages.

Proximate analyses of the hays used in the feeding and digestion trials are also presented in Table 1. One may note that hay was generally characterized by lower ether extract and crude fiber and higher NFE values than silage, particularly direct-cut silage.

TABLE 1

Chemical composition of crops as stored and as removed from storage [a]

	Dry matter	Dry matter constituents						
		Crude protein	Ether extract	N-free extract	Crude fiber	Ash	Sugar [b]	Carotene
	(%)							(p.p.m.)
Experiment 1								
As stored								
Direct-cut silage	26.6	20.7	2.4	43.2	24.8	8.9	3.3	215
Haylage	50.6	20.6	2.2	43.5	25.7	8.0	3.5	144
As removed								
Direct-cut silage	27.1	21.1	3.2	39.0	27.3	9.4	0.1	133
Haylage	53.1	20.0	2.4	41.3	27.7	8.6	2.0	35
Hay	89.9	18.7	2.0	46.2	25.9	7.2		
Experiment 2								
As stored								
Direct-cut silage	20.5	20.0	1.9	41.4	28.5	8.2	4.6	183
Haylage	43.7	18.4	1.5	40.9	30.6	8.6	5.1	125
As removed								
Direct-cut silage	24.0	18.8	2.4	37.4	33.2	8.2	0.1	186
Haylage	43.7	18.6	2.3	38.0	31.3	9.8	2.5	81
Hay	89.3	18.2	1.5	40.1	30.8	9.4		
Experiment 3								
As stored								
Direct-cut silage	22.5	18.5	1.8	38.3	34.4	7.0	5.4	170
Haylage	38.9	17.5	1.6	37.8	36.1	7.0	4.3	132
As removed								
Direct-cut silage	26.7	17.5	2.5	35.0	37.3	7.7	0.2	155
Haylage	39.1	17.7	2.5	38.0	34.0	7.8	1.2	82
Hay	88.9	16.4	1.5	38.9	35.6	7.6		

[a] Values for silages removed represent all good silage preserved. Values for hays represent only that part of the hay used in feeding and digestion trials.

[b] Total sugar expressed as glucose.

The average grade and classification of the hay samples as determined by the Inspection Branch, Agricultural Marketing Administration, were as follows: Experiment 1, U. S. No. 2 Alfalfa Hay; Experiment 2, Sample Grade Alfalfa Light Grass Mixed Hay, and Experiment 3, U. S. No. 2 Alfalfa Light Grass Mixed Hay. The Experiment 1 hay fell below the No. 1 requirement by a small amount of leaf, and Experiment 2 hay was graded as Sample Grade because of the weed content. Presumably, leaf content of the silages was somewhat higher and proportional content of plant species about the same as the hays; however, these values were not determined.

Average chemical values descriptive of the type and extent of carbohydrate fermentation and protein breakdown are presented in Table 2. With the exception of haylage in Experiment 3, pH values were so nearly alike for all silages

TABLE 2

Chemical quality of silages after storage [a]

	pH	Ammoniacal nitrogen		Organic acids					
		As protein	As per cent of total	Acetic	Propionic	Butyric	Lactic	Total [b]	Lactic / Total
				(%)					
Experiment 1									
Direct-cut	4.87	3.4	16.1	5.6	0.4	0.6	2.2	8.8	.25
Haylage	4.94	1.9	9.5	1.2	0.1	0.1	1.3	2.7	.48
Experiment 2									
Direct-cut	4.94	3.9	20.7	5.5	0.8	1.2	0.9	8.4	.11
Haylage	4.89	2.0	10.8	1.5	0.1	0.1	2.2	3.9	.56
Experiment 3									
Direct-cut	4.94	3.4	19.4	6.2	0.7	2.2	1.2	10.3	.12
Haylage	4.54	2.1	11.9	3.4	0.1	0.1	4.5	8.1	.55

[a] Ammoniacal nitrogen and organic acids on dry matter basis.
[b] Summation of acetic, propionic, butyric, and lactic values.

that they gave little indication of differences in quality. However, rather consistent relationships between direct-cut silages and haylage can be noted with respect to the other quality criteria. About one half as much ammoniacal nitrogen was present in haylage as in direct-cut silage and it contained markedly less of each organic acid excepting lactic. Acetic acid was predominant in direct-cut silage while lactic was the predominant acid in all haylages.

Quality of the Experiment 1 haylage declined noticeably after the silo was about one-half emptied until it became so poor that it was termed spoilage. During this period no distinct trend in the values for organic acids was noted, but the amount of ammoniacal nitrogen expressed as protein increased from less than 2% to more than 5% of the dry matter and the pH increased from 4.9 to 5.9. The shift in these values as the upper layers of direct-cut silage was fed out was also noticeable but not nearly as marked.

Percentage losses and recoveries of dry matter are summarized in Table 3. Highest recovery for direct-cut silage was obtained in Experiment 1 when relatively little seepage occurred. Top spoilage of direct-cut silage occurred in

TABLE 3

Percentage recovery and loss of stored silage dry matter

	Experiment 1		Experiment 2		Experiment 3	
	Direct-cut	Haylage	Direct-cut	Haylage	Direct-cut	Haylage
Recovered as silage	93.9	55.9	77.4	88.9	75.6	96.0
Lost as:						
Spoilage	0	35.1	3.4	5.6	0	0
Liquid	6.1 [a]	0.0	9.7	0.0	7.3	0
Gas		9.0	9.5	5.5	17.1	4.0
Total	6.1	44.1	22.6	11.1	24.4	4.0

[a] Combined liquid and gas loss.

Experiment 2 only; however, total recovery was about equal in Experiments 2 and 3 because of higher gaseous losses in Experiment 3. The low recovery rate of Experiment 1 haylage was caused by a large amount of spoilage. This spoilage was atributed to leaks in the silo, so both silos were rebuilt previous to Experiment 2. Spoiled haylage was much reduced in Experiment 2, and was eliminated in Experiment 3 when the last two loads were stored in an unwilted condition. High recovery rates were obviously dependent on good control of both seepage and spoilage.

A summary of the animal data obtained from the feeding trials is presented in Table 4. The relationships appearing in the results of Experiment 1 differ

TABLE 4

Feeding trial results

	Feed D.M. per cwt.			FCM production		Liveweight	
	Forage	Grain	Total	Daily average	10-day regression	Average	10-day regression [a]
	(lb.)						
Experiment 1							
Direct-cut silage	2.30 [a]	.66	2.96	23.8 [a]	− .84 [a]	1,043	+16.2 [a]
Haylage	2.33 [a]	.58	2.91	23.5 [a]	−2.06 [c]	1,120	− 7.7 [a]
Hay	2.62 [a]	.44	3.06	26.3 [a]	−1.52 [b]	1,122	+ 9.6 [a]
Experiment 2							
Direct-cut silage	1.81 [a]	.66	2.47	24.6 [a]	−1.53 [b]	983	− 8.8 [b]
Haylage	2.16 [b]	.63	2.79	25.9 [a, b]	−1.05 [a]	1,026	+ 4.8 [a]
Hay	2.41 [c]	.61	3.02	27.1 [b]	−1.10 [a]	1,019	+ 7.8 [a]
Experiment 3							
Direct-cut silage	1.93 [a]	.42	2.35	18.0 [a]	−1.63 [a]	868	− 2.7 [b]
Haylage	2.10 [a]	.42	2.52	17.7 [a]	−1.15 [a]	892	− .7 [b]
Hay	2.36 [b]	.41	2.77	19.5 [b]	−1.33 [a]	890	+ 6.7 [a]

[a] Calculated from liveweights 10, 20, 30, and 40 days after ration change.

[a, b, c] Means within a group followed by different letters are different at the 5% level of probability (Duncan's Multiple Range Test).

distinctly from those of the subsequent experiments. Dry matter consumption rates were particularly high for all forages and the differences among them were rather small. The negative regression of FCM production on time was least for direct-cut silage and greatest for haylage. In Experiment 2, dry matter consumption from forage and FCM production were least on direct-cut silage and greatest on hay, with haylage values being intermediate. Forage dry matter consumption relationships were similar in Experiment 3 to those of Experiment 2. Milk production was higher and decline in production less for the hay than the direct-cut silage ration in both Experiment 2 and Experiment 3. Milk production from haylage approached that from hay in Experiment 2, but was similar to that from direct-cut silage in Experiment 3. Hay rations produced small weight gains and direct-cut silage marked losses in Experiments 2 and 3. Again, haylage occupied an intermediate position.

Mean values for the digestibility of forage dry matter and its constituents are presented in Table 5. The statistically significant differences among means, within years and over years, were estimated by separate and combined analyses

TABLE 5

Average digestion coefficients for forage components

	Dry matter	Crude protein	Ether extract	Nitrogen-free extract	Crude fiber	Ash
Experiment 1						
Direct-cut silage	53.4	58.5[b]	36.6[c]	60.3[a]	41.9	53.5[b]
Haylage	50.4	52.3[a]	15.7[b]	61.0[a]	39.3	42.4[a]
Hay	56.2	68.2[c]	−10.2[a]	67.1[b]	45.7	44.9[a]
Mean differences		**	**	*		*
Experiment 2						
Direct-cut silage	55.7[a]	67.1[b]	57.0[b]	54.8[a]	50.2	47.5
Haylage	56.0[a]	61.0[a]	57.1[b]	64.8[b]	41.2	49.9
Hay	61.4[b]	66.3[b]	34.0[a]	70.5[b]	49.8	47.9
Mean differences	*	**	**	**		
Experiment 3						
Direct-cut silage	55.6[b]	65.9[b]	63.3[b]	57.8[a]	48.5	38.5
Haylage	46.4[a]	54.1[a]	62.5[b]	52.3[a]	39.2	31.7
Hay	58.1[b]	67.7[b]	32.6[a]	67.3[b]	47.4	38.7
Mean differences	**	**	**	**		
Experiments 1, 2, and 3						
Direct-cut silage	55.0[b]	64.0[b]	53.2[c]	57.6[a]	47.0[b]	45.9
Haylage	50.6[a]	55.7[a]	46.4[b]	58.8[a]	39.8[a]	40.6
Hay	58.6[c]	67.4[c]	19.9[a]	68.2[b]	47.6[b]	43.4
Mean differences	**	**	**	**	**	

* Mean differences statistically significant at 5% probability.
** Mean differences statistically significant at 1% probability.
[a, b, c] Means within a group followed by different letters are different at the 5% level of probability (Duncan's Multiple Range Test).

of variance, respectively. Duncan's multiple range test was used to identify statistically significant differences between means at the 5% level of probability.

The digestibility of hay was highest, or not significantly different from the highest, with respect to dry matter, crude protein, and nitrogen-free extract in each year and over the 3 yr. Digestibility of ether extract was consistently lowest in hay.

Digestibility of haylage dry matter, protein, ash, crude fiber, and nitrogen-free extract was lowest, or not significantly different from the lowest, in each and over all years with one exception. In Experiment 2, haylage nitrogen-free extract digestibility was significantly higher than that fraction in direct-cut silage. Direct-cut silage, in general, occupied an intermediate position regarding the digestibility of dry matter and all constituents excepting ether extract.

DISCUSSION

The efficiency of preservation in gas-tight storage is of considerable economic importance, since part of the higher dollar cost of such storage as compared to conventional storage may logically be charged against any improvements in preservation. These data indicate that a loss of 4% dry matter is about the minimum to be expected from a haylage system. This might be compared to the minimum loss obtainable in conventional silos. A loss of 8% dry matter in wilted silage stored in a conventional silo has been reported by Gordon *et al.* (6). If one accepts these values as being near minimums for the respective

structures, the economic evaluation may be based on the dollar value of a 4% saving in loss of dry matter ensiled. Consideration should also be given to any differences in maintenance cost, labor cost, or feeding value which may be involved.

The lowered haylage recovery rates observed in Experiments 1 and 2 are obviously the result of operating the system at less than maximum efficiency. Difficulties with prevention of spoilage may arise as they did in these experiments, but should not be considered a necessary characteristic of the system.

It is generally agreed that high-quality silage is characterized by a low pH, low contents of butyric acid, acetic acid, and ammoniacal nitrogen and by high levels of lactic acid. Since a broad range of initial silage moisture contents was obtained in this study, it is interesting to observe the extent to which initial moisture level was associated with each of these quality criteria. The correlations of average per cent dry matter when stored with average values for organic acids, total acid, and ammoniacal nitrogen were calculated for the six silages. The sum of the four organic acid values was termed total acid. These correlation coefficients were as follows: acetic acid, $-.969^{**}$; propionic acid, $-.925^{**}$; butyric acid, $-.802$, lactic acid, $+.310$, total acid $-.893^{*}$, and ammoniacal nitrogen, $-.977^{**}$.

Significant correlations of biochemical values when using only six pairs of data are remarkable. The correlation for butyric acid failed to be statistically significant at 5% level by a very small margin. In effect, increases in dry matter content were significantly correlated with improvement in chemical quality as indicated by decreases in ammonia, acetic acid, propionic acid, butyric acid, and total acid. Lactic acid content and pH are sometimes used as the primary indicators of chemical quality. In these data, however, these values were relatively constant, although other criteria indicated a wide difference in qualities. Apparently, the fermentation of haylage is rather limited and is more conspicuously characterized by the absence of undesirable, rather than by the presence of desirable fermentation end products.

The regressions of these chemical fractions on per cent initial dry matter as the independent variable (X) have been plotted in Figure 1. It is here shown graphically that increased dry matter content tended to reduce the extent of all the biochemical changes being measured, with the exception of lactic acid, and that lactic acid represented an increasingly large proportion of the total acid developed, although it was never present in very large amounts. All of these changes indicate a positive relationship between per cent dry matter and chemical quality. The b values for these regressions were: acetic acid $-.172 \pm .022$, propionic acid $-.024 \pm .005$, butyric acid $-.055 \pm .021$, lactic acid $.033 \pm .051$, total acid $-.218 \pm .0548$, and ammoniacal nitrogen $-.371 \pm .040$.

The reasons for this marked effect of dry matter content on the biochemical development of silage are not known. However, the observations are in agreement with those of Wieringa (20), who has reported that increasing the osmotic pressure of the forage liquid phase by wilting or additions of salt, favor the development of a desirable fermentation by suppressing development of

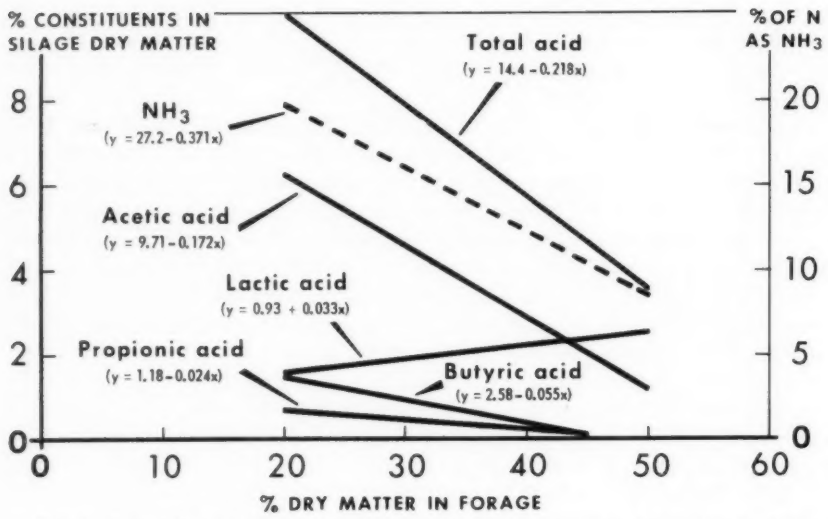

FIG. 1. Regressions of chemical constituents in silage on per cent dry matter in forages when stored.

butyric acid–forming bacteria. The osmotic pressure of the silage solution should be higher in the dried forages, although this was not determined in these experiments.

The final feeding value of each crop used was highest when preserved as barn-dried hay. Therefore, the extent to which the silages approached the hay in feeding value serves as a rather critical basis for evaluating the effects of ensiling procedures on feed value. When the feeding trial results from all 3 yr. are considered, the direct-cut silage was generally of lowest feeding value. The improvement realized from making haylage instead of direct-cut silage varied considerably between experiments, the improvement being most significant in Experiment 2 and least in Experiment 1. It should be kept in mind that the direct-cut second- and third-cutting forage used for Experiment 1 was relatively dry and resulted in better chemical quality than the other direct-cut silages (Table 2). The design of the feeding trial, as well as observed difficulties in preventing air leaks and spoilage in Experiment 1 haylage, also tended to distinguish this experiment from the others.

The correlations of silage dry matter consumption with the several measures of chemical quality were examined. Rate of dry matter consumption (pounds per 100 lb. liveweight) was chosen as a single measure of feeding value in this correlation, since there was a generally positive association of this value with milk production and live weight gain. Chemical values applying specifically to those portions of the silages used during the feeding trials were used in these correlations, rather than the average values presented in Table 2. The correlations of silage dry matter consumption per 100 lb. live weight with chemical

quality measurements were pH −.561; ammoniacal nitrogen expressed as percentage of total nitrogen, −.890*; acetic acid, −.723; propionic acid, −.909*; butyric acid, −.723; lactic acid +.362; total acid −.620; and per cent dry matter in silage, +.694. (Statistical significance at the 5% level is indicated by an asterisk and total acid equals the sum of the four acids measured.)

Variations existed between experiments with respect to level of milk production, stage of lactation, and grain supplementation of cows, as well as crop maturity and distribution of plant species. It is rather remarkable to observe the high correlations between consumption and some of the chemical constituents, in spite of these disturbing influences. The correlations indicate that highest animal acceptability is attained in silages having the lowest values for ammoniacal nitrogen and acetic, propionic, and butyric acid. It is clear from Table 2 that the haylage fits this description more closely than direct-cut silages. The effects of lactic acid content and pH on feeding value, as indicated by the correlations, appear relatively unimportant.

The digestibility coefficients clearly indicated that the nutrient value of the original crops was best preserved as barn-dried hay. The particularly low coefficients for ether extract in hay are not uncommon in hay rations and probably result from conversion of carbohydrates to ether-extractable materials during digestion. Such a conversion tends to lower apparent ether extract digestibility and increase apparent NFE digestibility. It is reasonable to expect less of this conversion in the digestion of silages, since considerable change of this kind has already occurred in the silo.

The generally lower digestibility of haylage can not be adequately explained, although some contributing factors may be suggested by the data. Since the digestibility of protein was the most seriously depressed fraction, the possibility of deleterious heating should be considered. Relatively dry silage of low density readily develops heat if exposed to air and it is known that some air did enter these silos. Shepherd (15) also mentioned some difficulty with heating under similar conditions. Unfortunately, no temperature measurements were made, but haylage was noticeably warmer than the silage when removed from the silo. The possibility that preferential loss of more digestible plant materials occurred during harvest of the haylage is not supported by the similarity of proximate analyses of direct-cut silage and haylage.

The true feeding value of forages is affected fully as much by acceptability as by nutrient content. Thus, dry matter intake (Table 4) times dry matter digestibility (Table 5) is an appropriate combined expression of potential feeding value, as proposed by Gordon *et al.* (6). The feed value potential in terms of pounds of digestible dry matter consumption per 100 lb. of live weight for direct-cut silages, haylage, and hay, respectively, were: Experiment 1, 1.23, 1.17, and 1.47; Experiment 2, 1.01, 1.21, and 1.48; Experiment 3, 1.07, 1.06, and 1.37. Expression of potential feeding values in this manner illustrates how the haylages, which were generally inferior in terms of digestible nutrient content, produced results equal to or better than those from direct-cut silages during the feeding trials.

These results indicate that the greater animal acceptability of haylage dry matter as compared to direct-cut silage was, to a large extent, balanced by a lower digestibility. Thus, realization of the maximum potential feed value of haylage necessitates that it be produced without reduction of digestibility coefficients. This probably means that air must be more adequately controlled than was done in these experiments.

Silos would be more rapidly fed out in many commercial installations than was possible under the conditions of this experiment. Such a practice would, from a practical standpoint, make air control less critical. The extent to which air exclusion is a problem in similar structures under farm conditions is purely a matter of speculation. However, if heating or visible top spoilage occurs, at least one of the following corrective measures should be taken: 1. Correct any detectable leaks in the structure, breather bag, or relief valve. 2. Feed at a faster rate. 3. Store forage at a higher moisture content.

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FACTORS NECESSARY FOR PREDICTING RESPONSES OF HEIFERS TO SILAGES OF VARYING QUALITY [1]

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SUMMARY

Three silages with calculated dry matter digestibilities of 56, 63, and 68% were individually fed to dairy heifers to study factors influencing average daily gain. The data were analyzed by least-squares analysis and showed significant influences for digestibility, dry matter intake, and gain/100 lb. of feed on average daily gain ($R^2 = .966$). Dry matter intake was influenced by digestibility, body weight, and average daily gain ($R^2 = .890$). The animals fed the 68% digestible silage had an average daily gain which was 207% of the gain of the heifers on the 56% digestible silage. The respective differences in digestible dry matter intake and Nutritive Value Index were 190 and 189%. A suggested program for obtaining reliable information to be used in forage testing programs is outlined.

Studies of the feeding value of forages have occupied the time and efforts of many research men for nearly a century. Today, there are published data covering in some cases nearly 50 components for some 4,000 forages (4), as well as many publications with digestion coefficients. Despite all this work, a research worker handed a sample of forage must still make a somewhat wild guess as to its feeding value. The recent introduction of forage testing programs in several states has drawn sharp attention to this problem. In the forage testing program, the usual procedure is to calculate the digestible dry matter or total digestible nutrients from the content of crude fiber. Current methods for calculating dry matter digestibility are far more accurate than are methods for predicting dry matter intake and animal performance. Although the programs have been generally successful in correcting gross deficiencies in dairy feeding programs, their future usefulness depends upon the availability of more accurate methods for predicting the feeding value of forages. Crampton *et al.* (2) suggested a means for combining voluntary intake and digestibility into a single numerical value which they termed the Nutritive Value Index (N.V.I.). This index was found to be highly correlated with body weight changes in the order of $r = 0.88$ to 0.94. Since the above work was done with sheep, it seemed desirable to determine the usefulness of the N.V.I. for growing dairy heifers.

EXPERIMENTAL PROCEDURE

Animals. The animals used in the feeding trials were Guernsey and Holstein heifers from the station herd. In each trial, animals ranging from 200 to 600 lb. were used to permit a study of the influence of animal size on intake and gain in weight. The heifers were individually fed a daily ration of silage sufficient to permit a weigh-back of about 10% of the quantity consumed. Each

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feeding period consisted of a seven-day preliminary and 42-day experimental period. The animals were weighed at 1:30 P.M. every seventh day. This procedure was expected to give a standard error of average daily gain of about ±.14 lb. when at least ten heifers per treatment were used [Bailey *et al.* (1) and McCullough and Neville (3)]. In the feeding trials, ten heifers were fed the lowest quality silage (oats) and 12 heifers were used on each of the other silages.

Silages. The data on intake and gain are from three silages feeding trials representing silages with a range in apparent dry matter digestibility from 56 to 68%. Dry matter digestibility was calculated by the regression $Y = 87.6 - 0.81\ X$ where X is crude fiber of the silage dry matter. This formula was found to predict the dry matter digestibility of 38 grass and grass-legume silages with a standard error of ± 2.08 units. The formula slightly underestimated digestibility by 0.56 unit. The 56% silage was an oat silage cut at the heading stage, whereas the 63 and 68% silages were made from alfalfa cut at the early bloom stage. The 63% silage was preserved with molasses and the 68% with ground snap corn.

Statistical analysis. The data were analyzed by least-squares multiple regression utilizing the model:

$$Y_1 \text{ or } Y_2 = b_o + ai + bX_1 + bX_2 + bX_3 + bX_4 + bX_5$$

where: b_o = a value common to all animals when the sum of each set of discrete variables and all regression variables are zero; ai = a constant for the i^{th} digestibility; X_1 = net partial regression of body weight of animals on silage with 56% digestibility. This applied only to calves on this silage and bX_2 and bX_3 would be deleted for this silage. Similar application applies to bX_2 and bX_3 regressions. X_2 = net partial regression of body weight of animals on silage with 63% digestibility; X_3 = net partial regression of body weight of animals on silage with 68% digestibility; X_4 = net partial regression of dry matter intake; X_5 = net partial regression of pounds of gain per 100 lb. feed; Y_1 = average daily gain: and Y_2 = dry matter intake.

RESULTS AND DISCUSSION

The influence of the silages on dry matter intake and average daily gain is shown in Table 1. Dry matter intake increased from 7.4 lb. on the 56% digestible silage to 11.3 lb. on the 68% silage, and was accompanied by an increase in gain from 0.93 to 1.81 lb. per day. The correlation between intake and aver-

TABLE 1

Mean intake, body weight and gain for the silages

Silage	Body weight	Dry matter intake	Gain/100 lb. dry matter	Average daily gain
	(lb.)			
56% Digestibility	416	7.4	14.2	0.93
63% Digestibility	482	10.9	12.6	1.34
68% Digestibility	394	11.3	16.0	1.81

age daily gain was .638. The efficiency of gain varied with the ration and did not follow a straight line. This was probably due to such influences as level of feeding and ration balance. Within the silages, the correlation between gain/100 lb. feed and average daily gain was .803.

TABLE 2

Influence of the variables on average daily gain (Y_1) and dry matter intake (Y_2)

Variable	Av. daily gain	Dry matter intake
Constants		
56% Digestibility	−.1653*	−2.1168*
63% Digestibility	+.0296*	+ .5632*
68% Digestibility	+.1357*	+1.5356*
Body weights		
56% Digestibility (X_1)	.0006	.0114**
63% Digestibility (X_2)	.0006	.0099**
68% Digestibility (X_3)	.0003	.0096**
Average daily gain (X_4)	.....	1.5531**
Dry matter intake (X_4)	.0982**	.....
Gain/100 lb. feed (X_5)	.0896**	.1110*
b_o	−.9899	4.8845
R^2	.966 **	.890 **

* Significant at 5% level of probability.
** Significant at 1% level of probability.

In Table 2 are shown the relationships between average daily gain (Y_1) and dry matter intake (Y_2). The constants for the three silages are of interest, since they measure the net effect of the silages with the other variables being held constant. The animals on the intermediate quality silage had an increase in gain of .19 lb. per day and those on the high-quality silage an increase of .30 lb. per day due entirely to change in silage digestibility. The remainder of the difference in average daily gain was due to the increase in intake which accompanied the increase in dry matter digestibility. Nonsignificant regressions for body weight indicate that size of animal was unimportant in average daily gain on the silages. Over-all, differences in dry matter intake and gain/100 lb. feed were significant influences on average daily gain. The R^2 of .966 indicates that the variables studied explained 96% of the variation in average daily gain. The data indicate that differences of five percentage points in dry matter digestibility may exert significant differences in average daily gain in dairy heifers.

The model employed accounted for 89% of the variation in dry matter intake ($R^2 = .890$). The significant factors influencing intake were body weight, efficiency of gain, and digestibility. Of particular interest was the increase of 3.6 lb. in dry matter intake which accompanied a change in digestibility from 56 to 68%. The high R^2 of .96 and .89 are of particular interest, since the data used were from silages of different species and ensiled with different preservatives. If this holds true for other experiments, it may be possible to develop single prediction equations to cover several forages.

To study the relative influence of the silages in animal performance, the data were recalculated in the regression formulas in terms of a 400-lb. animal. This permits a comparison of the silages at a constant level of nutritive use for maintenance. The results are shown in Table 3. The relative gain of the animal

TABLE 3

Summary of the data calculated from the regression formulas in terms of a 400-lb. heifer

Silage	Body weight	Digestible dry matter intake	Gain/100 lb. dry matter	NVI [a]	Average daily gain
	(lb.)	*(lb.)*	*(lb.)*		*(lb.)*
56% Digestibility	400	4.1	14.2	47	0.87
63% Digestibility	400	6.9	12.6	79	1.17
68% Digestibility	400	7.8	16.0	89	1.81
Relative change:	68% dig. / 56% dig.	190%		189	207%

[a] Crampton's Nutritive Value Index.

on the 68% digestible silage was 207% that of the 56% digestible silage. The relative changes in digestible dry matter intake and Nutritive Value Index were 190 and 189%. Thus, the magnitude of the change in average daily gain due to increasing forage quality was adequately described by either digestible dry matter intake or the Nutritive Value Index.

In light of these findings, it would appear possible to establish a simple research program to provide more accurate information for predicting animal response to various qualities of forage. The program should include the following:

(1) Conduct a series of feeding trials using widely varying samples of commonly fed forages. The trials should include at least eight animals per treatment, which are individually fed for a minimum of 42 days.

(2) Digestibility studies should be included.

(3) From the data obtained, a series of nutritive value indexes can be obtained for which animal gain data are also available.

(4) From the above data, prediction formulas, based on chemical composition, can be prepared from which predicted intake and animal response can be calculated for samples of similar materials.

The type of information which will be made available should permit sounder recommendations to dairy farmers in using their available feed supplies.

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EFFECT OF A NEW ANTIBIOTIC, SPIRAMYCIN, ON YOUNG DAIRY CALVES [1, 2, 3]

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SUMMARY

Thirty-six newborn male Holstein and Jersey calves were divided at random into three groups according to breed. Group I served as the control and was fed an unsupplemented calf starter. Group II was fed 250 mg. CTC for the first three days of life, 50 mg. for the next 28 days during the milk feeding period, and also a CTC-supplemented calf starter (18.6 mg. CTC per lb.). Group III was fed 250 mg. spiramycin for the first three days of life, 50 mg. for the next 28 days, and a spiramycin-supplemented calf starter (18.6 mg. per pound). Beginning at four days of age, whole milk was fed to all calves at the rate of 10% of the body weight daily for 28 days. Good-quality alfalfa hay and calf starter were also fed, beginning on the fourth day of life.

At 12 wk. of age, the two antibiotic groups of calves had achieved a significantly ($P < .05$) larger body weight gain than the control calves, by approximately 22%. A comparison between the gains of the two antibiotic groups showed no statistical difference. Feed conversion data in therms of E.N.E. required per pound of gain in body weight were in favor of the antibiotic-fed groups over the control, but this difference was not statistically significant.

The calves in the two antibiotic-supplemented groups had less severe periods of scours, which were found to be highly significant ($P < .01$) over the controls.

Spiramycin is of nutritional value for young calves as it promotes growth and reduces scours and compares favorably with CTC.

Since 1950, many research workers have confirmed the beneficial results of chlortetracycline (aureomycin) supplementation in the young dairy calf's ration on increased growth rate, reduced incidence of diarrhea, and general health of the calf (1, 2, 5, 6, 11, 12, 16, 17). Reports of antibiotic research in calf nutrition up to 1955 have been reviewed by Lassiter (6) and Reid *et al.* (12).

The relative nutritional value of other antibiotics for young calves have also been investigated, but to a lesser degree than chlortetracycline (2, 4, 6-8, 10, 12, 13, 18). A few reports have been concerned with the value of mixtures of certain antibiotics (2-4, 8, 14).

The apparent value of spiramycin supplementation to young dairy calves has not been reported. The purpose of this experiment was to study and compare the effect of spiramycin to chlortetracycline (CTC) on weight gains, feed conversion, incidence and severity of scours, and calfhood fatalities.

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[1] Supported in part by a grant from Rhodia, Inc., New York, New York.

[2] The data are from a thesis presented by the senior author to the graduate faculty of Louisiana State University in partial fulfillment of the requirements for the M.S. degree.

[3] Presented in part at the 55th Annual Meeting of the American Dairy Science Association, Logan, Utah, June, 1960.

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This experiment was conducted at the Louisiana State University Dairy Calf Nutrition barn during the months of September, 1959, to March, 1960. Thirty-six newborn male Jersey and Holstein calves were divided at random and placed into three groups according to breed, at one to three days of age as they became available. The dietary treatments of the three groups were as follows: Group I served as the control and received no antibiotic supplement during the milk feeding or in the basal starter ration. Group II received a 250-mg. capsule of crystalline chlortetracycline (CTC) per calf daily for the first three days of life. For the next 28 days during the milk feeding period, each calf in this group received a 50-mg. capsule of crystalline CTC daily. On the fourth day of life, the calves were offered a CTC-supplemented starter ration containing 18.5 mg. CTC per pound of starter, which was fed for the duration of the experimental period. Group III received a 250-mg. capsule of crystalline spiramycin per calf daily for the first three days, and for the next 28 days each calf received a 50-mg. capsule of crystalline spiramycin per calf daily. A spiramycin-supplemented starter which contained 18.6 mg. crystalline spiramycin per pound of starter was offered the calves in this group on the fourth day of age and fed for the duration of the trial. All calves were kept on sugar cane bagasse bedding, in individual stalls. Each calf was observed three times daily for the possible occurrence of scours, respiratory disease, or physical defects. The calves were weighed weekly up to 12 wk. of age.

All calves on the experiment received colostrum for the first three days of life. Colostrum was fed at the rate of 10% of body weight, and this amount was divided equally into two parts, making up the morning and evening feedings. Whole milk feeding began on the fourth day of life and continued at the 10% rate for the next 28 days. The only time feeding of the milk deviated from the normal rate was when a calf had scours. At these times, the amount of milk at each feeding was reduced 30-50%, depending on the severity of the scours. This was the only manner in which scours was treated.

The calf starters were offered to the calves in each group at a maximum rate of 5 lb. per day for the Holstein calves, and 4 lb. per day for the Jersey calves. The composition of the calf starters and the proximate analysis are shown in Table 1. The forage used in this experiment was good-quality alfalfa hay, which was placed before the calves beginning on the fourth day of age and fed ad libitum throughout the trial. Feed consumption was recorded daily. All data were subjected to statistical analyses, using the method of unweighted means (15).

RESULTS AND DISCUSSION

The average birth weights of the calves in each group and average gains adjusted for initial birth weights at 12 wk. of age are shown in Table 2. Six calves died during the course of the experiment and the data of the surviving calves were used for statistical analyses. Covariance analyses, using birth weight as the independent variable, indicated that the calves in the antibiotic groups (II and III) grew at a faster rate than those of the control group. The percentage increase in weight gain of the antibiotic groups over the control at 12 wk.

TABLE 1

Composition of calf starters[1]

Ingredient	Group		
	Control	Chlortetracycline	Spiramycin
	(lb.)		
Rolled oats	15.0	15.0	15.0
Cotton seed meal	35.0	35.0	35.0
Soybean oil meal	5.5	5.5	5.5
Yellow corn meal	31.5	31.5	31.5
Wheat bran	10.0	10.0	10.0
Steamed bonemeal	2.0	2.0	2.0
Salt	1.0	1.0	1.0
Quadrex IV[a]	0.2	0.2	0.2
Aurofac A[b]	0.0	1.0	0.0
Spiramycin[c] *(g.)*	0.0	0.0	1.86 g.

[a] Vitamin A and D supplements—Contents: vitamin A, not less than 4,540,000 U.S.P. Units (1,363 mg.) per pound (10,000 units per gram); vitamin D_2, not less than 567,500 U.S.P. Units per pound (1,250 units per gram) NOPCO Chemical Co., Harrison, New Jersey.

[b] Aurofac A containing 1.86 g. of chlortetracycline per pound was supplied by Lederle Lab. Div., American Cyanamid Co., Pearl River, New York.

[c] Spiramycin, 1.86 g. per 100 lb. of starter.

[1] Proximate analysis of starter ration: crude protein—22.5%, crude fiber—8.4%, N.F.E.—50.5%, ash—6.2%, ether extract—1.9%, and moisture—10.5%.

of age amounted to 25.8% for the CTC group and 19.5% for the spiramycin group. The differences among treatments on a within breed basis were found to be significant ($P < .05$) when gains were adjusted for differences in birth weights. No significant differences between gains of the two antibiotic groups were obtained. The treatment × breed interaction was significant at $P < .05$ for gains at 12 wk. of age. The growth increase of the calves in both antibiotic groups over the controls at 12 wk. of age is similar to those reported previously with CTC (1, 2, 5, 6, 11, 12, 17).

The average consumption of milk, calf starter, and hay was greater and feed conversion apparently better in the antibiotic-supplemented groups than in the

TABLE 2

Average weight gains of calves

Group and treatment	Breed and No. of animals		Av. birth weight	Av. gain at 12 wk. of age adjusted for birth weight[a]			Increase due to antibiotic
			(lb.)	*(lb.)*			*(%)*
	Holstein	*Jersey*		*Holstein*	*Jersey*	*Combined*	
I Control	6	5	68	84.9	70.7	77.8	
II CTC	5	3	70	115.5	80.2	97.9*	25.8
III Spiramycin	7	4	78	98.5	87.4	93.0*	19.5

[a] Two Holsteins died in Group I, two Holsteins and one Jersey in Group II, and one Holstein in Group III.

* Significant difference at $P < .05$ over control group. No significant difference between antibiotic groups.

TABLE 3

Average feed consumption and feed conversion of dairy calves at 12 wk. of age

Group	Av. consumption per group			Feed conversion
	Whole milk	Calf starter	Hay	Av. E.N.E.[a] per pound gain
	(lb.)			*(therms)*
I Control	214.32	178.46	31.22	2.8
II CTC	230.68	204.06	55.94	2.1
III Spiramycin	239.57	190.58	32.82	2.2

[a] E.N.E.—estimated net energy.

control group, as shown in Table 3. Feed conversion was calculated as therms of estimated net energy (E.N.E.) per pound of gain. The E.N.E. input was calculated for the calves on the three treatments for the period 0-12 wk. Milk was given a value of 20 therms, calf starter a value of 68 therms, and hay a value of 40 therms of net energy per 100 lb. (9). No significant differences in feed conversion among the three groups were obtained. This suggests that the increased weight gains obtained by the antibiotic groups were due to the larger amount of feed consumed and not to the efficiency with which they utilized the feed. This observation is in agreement with previous work reported at this station (5) and at Iowa (1).

At an early age, scours became a major problem in this study. Apparently, an infectious scour organism was introduced into the barn, and normal management practices were unable to clear it. All outbreaks of scours occurred during the calves' milk feeding period, i.e., the first 31 days of life. The number of calves scouring per treatment and the number of days of scours are shown in Table 4. A Chi-square test for differences indicated that the antibiotic-supplemented groups tended to control the severity and duration of scours which was found to be significant at $P < .01$, but failed to reduce the incidence of the number of calves scouring. Owen *et al.* (10) found that whereas several antibiotics under tests increased the rate of body weight gains, they had little or no effect on the incidence and severity of scours. Hogue *et al.* (4) suggested that the effect of antibiotics on the incidence of scours and growth is, at least in part,

TABLE 4

Distribution of scours

Group	No. of calves scouring	No. of days scouring	No. of deaths to 9 wk. of age (not due scours)
I Control	8	48	2
II CTC	7	28**	3
III Spiramycin	6	21**	1

** Highly significant difference at $P < .01$ over control group.

independent. Thomas *et al.* (16) suggested that the major advantage of feeding antibiotics to dairy calves is the reduction in the incidence and severity of scours and not the temporary growth response.

During the course of the experiment, six calves died. All deaths occurred during the first 9 wk. of age. There were two deaths in the control group, three deaths in the CTC group, and one death in the spiramycin group. Immediately following each death, the carcasses were examined by a member of the University veterinary staff. There was an indication of a Salmonella infection and possibly a virus infection. The carcasses showed dehydrated tissues, gastric congestion, and edema of the lungs. Calves that died showed no noticeable physical symptoms or abnormalities prior to their deaths. No deaths could be attributed to scouring, nor was any calf scouring at the time of death.

From the results obtained in this study, it appears that spiramycin is of nutritional value for young calves as it promotes growth and reduces scours and compares favorably with chlortetracycline.

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REGRESSION FACTORS FOR PREDICTING A SUCCEEDING COMPLETE LACTATION MILK RECORD FROM PART LACTATION RECORDS

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SUMMARY

The age- and season-corrected monthly D.H.I.A. milk records of 9,036 cows in 374 New York herds, and the succeeding record of 2,358 of these cows in 226 herds, are used to develop regression factors for predicting a succeeding complete lactation milk yield from part lactation records. A single test-day record from the fourth, fifth, or sixth month of a lactation has a correlation of 0.50 with the succeeding complete lactation. This correlation is nearly as large as the correlation between two successive complete lactation yields, which is 0.55. A linear function of the first five monthly test records predicts a complete second record as accurately as the total yield of the first lactation. Linear functions for the first seven monthly records are even more accurate. Prediction equations utilizing bimonthly test records are more highly correlated (0.57) with second complete records than complete first records, and are nearly as accurate as linear functions of ten monthly test-day records.

The ability to predict the succeeding record of a cow determines the success of herd-culling programs. Usually, the prediction is based on a cow's complete lactation record or on the average of her completed records. In actual practice, it would be useful to predict the succeeding record prior to completion of the current lactation. A cow could then be culled or saved at an earlier period, or future planning could be done at an earlier time. Such a prediction procedure would be especially valuable, if the accuracy of prediction on the basis of a linear function of part of the monthly records of a preceding lactation was greater than of prediction based on the total yield of the preceding lactation. The purpose of this study was to develop regression factors for predicting a succeeding complete milk record from various sets of sequential and cumulative monthly test-day milk records.

DATA

The D.H.I.A. milk records from 9,036 Holstein cows in 374 herds in five New York counties were used in this study. A succeeding second complete record was available from 2,358 of these cows in 226 herds. Ten monthly test-day records were available for each complete lactation, beginning with the first month of lactation and ending with the tenth month. A complete lactation record was regarded as the sum of the corresponding ten test-day records. The sample of monthly test records was taken from November, 1957, through December, 1959. Correction factors described by Van Vleck and Henderson (1) were used to adjust the monthly records for age at calving and season of calving.

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ANALYSIS AND CONSTRUCTION OF REGRESSION FACTORS

Estimates of within-herd components of variance and covariance were used in setting up the normal equations. The variances and covariances among the first records were estimated from a set of 9,036 records. The covariances between monthly test-day records of a current record and the complete succeeding record were estimated from a set of 2,358 records. The normal equations were solved to obtain the regression coefficients associated with various combinations of monthly records. The multiple correlation coefficient was chosen as the criterion for measuring the accuracy of prediction.

RESULTS AND DISCUSSION

The means of monthly test-day records for cows 72-77 mo. of age at calving and calving in Season 1 (December through March) are given in Table 1. These

TABLE 1

Means of monthly test-day records for cows calving at 72-77 mo. of age in Season 1 (December-March)

	Month										
	1	2	3	4	5	6	7	8	9	10	Total
Milk	57.1	56.4	53.6	49.5	45.5	40.5	34.3	27.4	18.2	11.8	394.3
Fat	2.26	2.00	1.84	1.71	1.56	1.41	1.21	1.00	0.68	0.44	14.11

are the base means to which the other monthly records were adjusted. Some of the examples which are presented later also make use of this table. Estimates of average monthly production can be obtained from these means if they are multiplied by the number of days in the average D.H.I.A. month, which is 30.5.

The single monthly test days which are the best for predicting the complete yield of a succeeding lactation are in the fourth, fifth, or sixth months. The correlations between these test records and the subsequent complete yield are 0.50. This correlation is somewhat surprising, since the usual method of prediction has a correlation of only 0.55 with the actual subsequent total yield (see Table 5). The usual procedure estimates the succeeding complete yield from the total production of the previous lactation. The regression coefficients for predicting a second complete record from a single monthly test-day record are shown in Table 2. As judged by their correlations with a complete succeed-

TABLE 2

Regression factors for estimating the succeeding complete lactation yield from a single monthly test record of the current lactation

	Month of lactation									
	1	2	3	4	5	6	7	8	9	10
b[a]	2.72	2.75	3.17	3.59	3.89	3.99	4.09	3.34	2.28	1.42
R[b]	0.36	0.42	0.49	0.50	0.50	0.50	0.49	0.41	0.30	0.20

[a] b is the regression coefficient associated with the monthly record.
[b] R is the correlation between the predicted and actual yield.

ing record, the third to the seventh months are about equally accurate in the prediction of a succeeding complete record. The prediction equations are of the form

$\hat{y} = \mu_y + b_i(X_i - \overline{X}_i)$ where

$\hat{y}$ is the predicted second record (age and season corrected),

μ_y is the herd mean of age- and season-corrected complete records,

b_i is the regression coefficient associated with the i^{th} month of lactation,

X_i is the age- and season-corrected test-day record for the i^{th} month of lactation, and

$\overline{X}_i$ is the age- and season-corrected herd mean of records of the i^{th} month of lactation.

As an example, suppose that the fourth monthly test record of a cow is 69.5 lb. of milk and that the age- and season-corrected herd averages are the means given in Table 1. Then, the estimate of the cow's next complete record is

$$\hat{y} = 394.3 + 3.59\ (69.5 - 49.5) = 466.1.$$

Multiplied by 30.5 the estimate of the next 305-day record is 14,216 lb. of milk.

Sequential monthly test data when used in multiple regression prediction equations will be more accurate than individual monthly records in predicting a succeeding record. These prediction equations are such that

$$\hat{y} = \mu_y + \sum_{i=1}^{m} b_i(X_i - \overline{X}_i) \text{ where } 1 \leqslant m \leqslant 10.$$

The regression coefficients for the sequential monthly records are given in Table 3, together with the multiple correlation coefficients. A linear function of the first five monthly records provides an estimate of a complete second record which is as accurate as the usual prediction method. If more monthly records are included, the accuracy is even better than the usual estimation procedure, although only slightly better. The correlation increases slowly, reaching 0.57 when all ten months are used in a multiple regression equation.

TABLE 3

Regression factors for estimating the succeeding complete lactation yield from sequential test-day data of the current lactation

Sequential months	Monthly regression coefficients										R
	1	2	3	4	5	6	7	8	9	10	
1	2.72	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.36
2	1.26	2.09	.....	.....	.....	.....	.....	.....	.....	.....	0.45
3	1.06	0.12	2.59	.....	.....	.....	.....	.....	.....	.....	0.50
4	0.93	−0.15	1.28	2.11	.....	.....	.....	.....	.....	.....	0.53
5	0.81	−0.18	1.13	0.90	1.95	.....	.....	.....	.....	.....	0.55
6	0.80	−0.19	1.10	0.69	0.86	1.65	.....	.....	.....	.....	0.56
7	0.86	−0.14	1.04	0.62	0.66	0.58	1.64	.....	.....	.....	0.57
8	0.87	−0.12	1.04	0.60	0.62	0.54	1.26	0.53	.....	.....	0.57
9	0.87	−0.12	1.04	0.60	0.63	0.54	1.27	0.46	0.08	.....	0.57
10	0.88	−0.12	1.03	0.60	0.63	0.54	1.27	0.46	0.02	0.06	0.57

In our example, suppose that the first five monthly age- and season-corrected records are 77.1, 76.4, 73.6, 69.5, and 60.5 lb. of milk, respectively. Then

$$\hat{y} = 394.3 + 0.81\ (77.1 - 57.1) - 0.18\ (76.4 - 56.4) + 1.13\ (73.6 - 53.6) + 0.90\ (69.5 - 49.5) + 1.95\ (60.5 - 40.5) = 486.5$$

Multiplying by 30.5, the predicted second record is 14,838 lb. of milk.

TABLE 4

Regression factors for estimating the succeeding complete lactation yield from sequential monthly test-day data of the current lactation when first months are missing

	Monthly regression coefficients										
	1	2	3	4	5	6	7	8	9	10	R
1	.....	.....	.....	.....	.....	.....	.....	.....	.....	1.42	0.20
2	.....	.....	.....	.....	.....	.....	.....	.....	2.52	−0.28	0.30
3	.....	.....	.....	.....	.....	.....	.....	3.70	−0.38	−0.07	0.41
4	.....	.....	.....	.....	.....	.....	3.53	0.85	−0.18	−0.01	0.49
5	.....	.....	.....	.....	.....	2.31	1.68	0.61	−0.15	0.05	0.52
6	.....	.....	.....	.....	1.99	0.91	1.39	0.44	−0.08	0.07	0.54
7	.....	.....	.....	1.65	0.87	0.65	1.26	0.41	−0.03	0.06	0.56
8	.....	.....	1.22	0.67	0.74	0.63	1.20	0.46	0.01	0.01	0.57
9	.....	0.24	1.07	0.63	0.72	0.61	1.21	0.47	0.01	0.01	0.57
10	0.88	−0.12	1.03	0.60	0.63	0.54	1.27	0.46	0.02	0.06	0.57

Multiple regression factors for the situation when the first months of records are lacking, as often is the case when herds enter central processing, are given in Table 4. The prediction equations now have the form

$$\hat{y} = \mu_y + \sum_{i=n}^{10} b_i\ (X_i - \overline{X_i}) \text{ where } 10 \geqslant n \geqslant 1.$$

Cumulative monthly test-day records would be more practical for use by records centers in predicting a second record than the use of multiple factors. Fewer factors are required and it is easier for records processing centers to carry forward cumulative production records rather than monthly records. The accuracy of prediction, however, is less. Five months of cumulative information are only as accurate as the single fourth, fifth, or sixth monthly records. Maximum accuracy of prediction from cumulative records is reached in 8 mo., when the correlation coefficient is 0.55, the same as for using total lactation yield to predict a succeeding complete record. The regression factors for cumulative test-day records are given in Table 5 for cumulative records when the last months of lactation are not available and in Table 6 for cumulative records

TABLE 5

Regression factors for estimating the succeeding complete lactation yield from cumulative test-day records of the current lactation

	Cumulative month of lactation									
	1	2	3	4	5	6	7	8	9	10
b	2.72	1.71	1.20	0.94	0.81	0.72	0.65	0.60	0.56	0.53
σ_b	0.16	0.08	0.05	0.04	0.03	0.02	0.02	0.02	0.02	0.02
R	0.36	0.44	0.47	0.49	0.51	0.53	0.54	0.55	0.55	0.55

TABLE 6

Regression factors for estimating the succeeding complete lactation yield from cumulative test-day records of the current lactation—first months missing

	Cumulative months of lactation									
	10	9	8	7	6	5	4	3	2	1
b	1.42	1.20	1.09	1.03	0.93	0.83	0.73	0.63	0.56	0.53
R	0.20	0.29	0.36	0.43	0.48	0.51	0.52	0.53	0.54	0.55

when the first months of lactation are missing. The equations for the former situation have the form

$$\hat{y} = \mu_y + b_j \sum_{i=1}^{j} (X_i - \overline{X}_i) \text{ where } 1 \leqslant j \leqslant 10 \text{ and}$$

b_j is the regression coefficient associated with the first j months of cumulative production. The latter situation has the prediction equations

$$\hat{y} = \mu_y + b_k \sum_{i=11-k}^{10} (X_i - \overline{X}_i) \text{ where } 1 \leqslant k \leqslant 10 \text{ and}$$

b_k is the regression coefficient associated with the last k months of cumulative production. Eight months of cumulative production are as accurate in predicting a second record as a complete first record.

Multiple regression equations for bimonthly test-day records used in predicting a second lactation are more accurate than the preceding total 305-day record. They are almost as accurate as regression equations utilizing all ten monthly records. This high degree of accuracy (0.57 and 0.56) demonstrates the validity of bimonthly testing programs. The trimonthly testing systems also are as accurate as the usual method of predicting a second record. The correlations with a second record are 0.56, 0.54, and 0.55 for the three trimonthly sets, respectively. The multiple regression factors for the bimonthly and trimonthly test-day records are shown in Table 7.

Cumulative bimonthly or trimonthly test-day records would be practical for use in predicting second records. The regression factors for estimating second records from these cumulative records are given in Table 8. As expected, the correlations are not as large as with the use of multiple factors.

TABLE 7

Regression factors for estimating the succeeding complete lactation yield from sequential bimonthly and trimonthly test-day data of the current lactation

Monthly set	Month of lactation										R
	1	2	3	4	5	6	7	8	9	10	
1	0.88	.....	1.25	.....	1.11	.....	1.80	.....	0.30	.....	0.57
2	.....	0.73	.....	1.53	.....	1.60	.....	1.02	.....	0.01	0.56
1	1.08	.....	.....	1.79	.....	.....	2.36	.....	.....	.....	0.56
2	.....	1.19	.....	.....	2.22	.....	.....	1.40	.....	.....	0.54
3	.....	.....	1.88	.....	.....	2.28	.....	.....	0.56	.....	0.55

TABLE 8

Regression factors for estimating the succeeding complete lactation yield from cumulative bimonthly and trimonthly test-day records of the current lactation

	Bimonthly set		Trimonthly set		
	1	2	1	2	3
b	1.06	0.99	1.62	1.50	1.54
R	0.55	0.53	0.54	0.52	0.52

CONCLUSIONS

Part lactation records are very valuable for predicting total milk production during a succeeding lactation. A single test-day record from the third, fourth, fifth, sixth, or seventh month of a lactation is nearly as accurate in predicting a succeeding complete record as the total yield of the current lactation. This result indicates that a farmer can cull cows on the basis of a single monthly test (as early as the third or fourth month of lactation) with almost as much success as culling on the basis of the cumulative 10-mo. records. Prediction equations involving multiple regression factors for monthly production will do a slightly better job of selecting cows for future production than will the usual selection procedure, which is based on total lactation records. The first five monthly records used in a multiple regression equation are as accurate as the cumulative 10-mo. total. The addition of the sixth- and seventh-month records to the equations improves the accuracy of prediction slightly more. The use of more than seven or eight monthly records in predicting a succeeding complete 10-mo. record does not appear to be warranted, since the increase in accuracy of prediction is slight.

Bimonthly testing seems to be justified for the prediction of future complete record production as well as for estimating the current complete lactation yield (2). Linear functions of either of the bimonthly testing possibilities will predict a succeeding complete record more accurately than will a complete cumulative record. Both bimonthly testing systems will do nearly as well as any linear combination of the ten monthly tests for predicting a second record. Linear functions of trimonthly tests are equally as accurate in prediction of a second record as the usual procedure.

The accuracy of predicting future production from linear functions of monthly test-day data indicates the possible use of part lactation records in the central processing of dairy records and for herd selection procedures. Linear functions of bimonthly or trimonthly tests are correlated closely enough with actual production and also predict future production accurately enough to be used by dairy records processing centers, if it is desirable to conserve time and to decrease the cost of testing programs.

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IMPROVEMENT IN PRODUCTION OF NEW YORK HOLSTEINS DUE TO ARTIFICIAL INSEMINATION

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SUMMARY

The first-lactation milk and fat records of 24,995 artificially sired progeny and 32,831 naturally sired progeny were compared on a within herd-year-season contemporary comparison basis over a period of nearly 10 yr. The artificially sired progeny were superior in every year of calving from 1951 through 1959, for milk and fat production. The trend in superiority was generally upward, although the low point was in 1953, followed by an increase and then a slight decline until 1959, when the superiority was greatest. Evidently, more emphasis was placed on selection of sires according to fat production of progeny than milk production. Biases in estimation of superiority of artificially sired daughters would result if over-all season averages of artificially sired and naturally sired daughters were used.

Artificial insemination of dairy cows has received wide acceptance in recent years. In New York, in 1960, more than half of the dairy cow population was bred artificially, Krumm (7). However, the question is often asked "What improvement in production has resulted from artificial insemination?" Robertson and Rendel (9) proposed a method for determining the difference between first-lactation daughters of sires used in artificial insemination (AI) and first-lactation natural-service daughters. These workers (9) reported a significant advantage for AI daughters of the British Friesian breed and slight but non-significant disadvantages for the Shorthorn and Guernsey breeds. The New Zealand Dairy Board (8) reported a superiority of 10 lb. fat for 2-yr.-old artificially sired progeny over their naturally sired herd-mates. Several workers, using the contemporary comparison procedure of Robertson and Rendel (9), have estimated differences between AI and non-AI progeny for United States data—Wadell and McGilliard (13) for Michigan herds; Tucker and Farthing (11) and Tucker, Legates and Farthing (12) for North Carolina data; Hahn, Carmon, and Miller (6) for Georgia herds; and Guderyon, Corley, and Duckwall (5) for Wisconsin records. Most of these studies, except for the last cited, have dealt with relatively few records. None has shown the trend of differences between AI and non-AI progeny over a period of years.

The purpose of this study was to compare the first-lactation records of New York Holstein AI and non-AI progeny within the same herd-year-seasons over a period of nearly 10 yr., in an attempt to learn if artificial insemination has improved the dairy cattle population in New York.

DATA

The 305-day, 2 ×, M.E. milk and fat records of 597,388 separate lactations of Holstein cows which calved between September 1, 1950, and November 30,

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1959, were available in the New York Dairy Records Processing Laboratory. Of these, only first-lactation records were used which were made in herd-year-seasons in which both AI and non-AI progeny had first records. The paper of Fritz, McGilliard, and Madden (4) indicated that few errors of classification would occur if first records were defined to be those initiated prior to 35 mo. of age. Since the lactation number was not recorded for the records, this criterion was used to distinguish between first and later lactation records. The number of AI and non-AI progeny records satisfying these requirements were 24,995 and 32,831, respectively. All first-lactation records were adjusted to a mature equivalent basis, since the range in age at calving for the first lactation would be at least 14 mo.

ANALYSIS PROCEDURE

The contemporary comparison procedure described by Robertson and Rendel (9) in Great Britain, and also later by Tucker *et al.* (12) in the United States, was used. The comparisons between first-lactation records of AI and non-AI progeny were made within herd-year-season groups. The differences were weighted by the reciprocals of their variances (ignoring sire variance) and summed over all herds in the particular year-season. This sum was then divided by the sum of the weights, to obtain the estimated difference between records of AI and non-AI progeny for the year-season. The year-seasons used are those that have been set up by the New York Dairy Records Processing Laboratory and are shown in Table 1. The estimates for the year-seasons were then pooled to obtain yearly estimates by weighting according to the sum of the weights for each year-season. Season 50-2 was included in the estimate for 1951.

TABLE 1

Year-seasons of calving used in the New York Dairy Records Processing Laboratory

Year-season	Inclusive months	Year-season	Inclusive months
50-2	Sept. 1950-Feb. 1951	56-1	Mar. 1956-Aug. 1956
51-1	Mar. 1951-Aug. 1951	56-2	Sept. 1956-Feb. 1957
51-2	Sept. 1951-Feb. 1952	57-1	None
52-1	Mar. 1952-Aug. 1952	57-2	Mar. 1957-July 1957
52-2	Sept. 1952-Feb. 1953	57-3	Aug. 1957-Nov. 1957
53-1	Mar. 1953-Aug. 1953	58-1	Dec. 1957-Mar. 1958
53-2	Sept. 1953-Feb. 1954	58-2	Apr. 1958-July 1958
54-1	Mar. 1954-Aug. 1954	58-3	Aug. 1958-Nov. 1958
54-2	Sept. 1954-Feb. 1955	59-1	Dec. 1958-Mar. 1959
55-1	Mar. 1955-Aug. 1955	59-2	Apr. 1959-July 1959
55-2	Sept. 1955-Feb. 1956	59-3	Aug. 1959-Nov. 1959

The contemporary comparison procedure should effectively remove herd, year, and season effects from the estimates of AI superiority. Since only first records are compared, the procedure also eliminates bias due to selection, if it can be assumed that no culling related to production is practiced prior to initiation of the first record. The Dairy Records Processing Laboratory extends all records which are incomplete. Therefore, culling on the basis of a short

record will not bias the estimates, if it can further be assumed that the extension factors are correct.

RESULTS

The estimated superiority by years of AI Holstein progeny calving in the period 1951 through 1959 above their non-AI contemporaries is presented in Table 2. The superiority is significantly greater (at the 5% or less significance

TABLE 2

Estimated superiority of first-record artificially sired (AI) Holstein daughters over first-record naturally sired (non-AI) daughters within the same herd-year-seasons

Year	Number of comparisons	Number progeny		Sum of weights	Superiority	
		AI	non-AI		Milk	Fat
					(lb.)	
1951	606	1,065	1,879	544.18[a]	262.3[b]	9.92[b]
1952	721	1,536	2,325	701.83	59.5	6.31[b]
1953	1,026	2,456	3,152	1,039.02	11.5	3.26
1954	1,134	2,831	3,903	1,225.00	284.2[b]	10.25[b]
1955	1,214	3,178	3,665	1,307.46	331.4[b]	11.34[b]
1956	1,176	2,983	3,663	1,248.14	169.2[b]	11.56[b]
1957	960	2,304	2,753	992.55	194.4[b]	8.43[b]
1958	1,866	4,710	6,630	2,106.86	103.3[b]	7.86[b]
1959	1,521	3,932	4,861	1,622.27	357.4[b]	17.62[b]

[a] The standard error of an estimate can be approximated by dividing the residual variance of milk records (4,500,000) or fat records (6,000) by the sum of the weights associated with each yearly estimate and then taking the square root of the result.
[b] Significant at the 5% level.

level) in all years except 1952 and 1953 for milk production and 1953 for fat production. There appears to be a cyclical pattern in the trend of differences. Superiority in 1951 is rather high, followed by a decline for 2 yr., after which a marked increase occurred in 1954. The superiority for 3 yr., 1954 through 1956, was relatively constant and then declined slightly for 2 yr. A marked upsurge then occurred for the last year of the study, 1959.

One of the more than 270 sires which contributed AI progeny to the New York Holstein population over the 10-yr. period had a marked impact on the production of AI progeny during the first 4 yr. of the 1950's. His estimated superiority by the contemporary comparison procedure is in Table 3, given for the years 1951 through 1954. Only a few of his progeny began a first record after 1954.

TABLE 3

Estimated superiority of first-record Dean daughters over first-record naturally sired daughters within the same herd-year-seasons

Year	Sum of weights	Superiority	
		Milk	Fat
		(lb.)	
1951	62.88	823.1	35.63
1952	62.79	1,031.5	51.58
1953	108.46	940.7	40.08
1954	90.85	811.5	40.36

The average 305-day, 2 ×, M. E. milk and fat records of all Holstein AI and non-AI sired cows contributing records to the New York Dairy Records Processing Laboratory for the second season of 1950 through the third season of 1959 are shown in Table 4. The seasonal fluctuations due to year and season of calving are readily apparent from examining the averages.

TABLE 4

Year-season averages of M.E., 2 ×, 305-day production records of artificially (AI) and naturally sired (non-AI) Holstein cows in New York Dairy Herd Improvement Associations

Year-season	AI progeny			non-AI progeny		
	No. of records	Milk	Fat	No. of records	Milk	Fat
		(lb.)			(lb.)	
1959-3	15,779	12,896	470.7	30,111	12,202	438.4
1959-2	4,206	12,470	452.5	14,810	11,901	428.6
1959-1	8,343	12,937	469.0	22,233	12,382	446.2
1958-3	12,489	12,792	466.5	28,345	12,417	448.0
1958-2	3,710	12,461	449.7	10,657	12,107	433.2
1958-1	7,325	12,808	461.5	18,962	12,531	447.4
1957-3	11,260	12,716	460.1	25,256	12,363	444.5
1957-2	5,058	12,216	439.7	15,009	11,855	423.8
1956-2	13,085	12,335	446.0	33,861	12,034	431.1
1956-1	5,431	11,909	430.6	17,045	11,570	415.4
1955-2	11,977	12,555	454.7	35,408	12,243	439.8
1955-1	5,319	12,091	435.4	18,274	11,699	416.5
1954-2	10,407	12,147	436.6	34,611	11,937	425.0
1954-1	4,033	11,710	422.4	16,238	11,483	409.2
1953-2	7,252	12,188	439.9	23,811	12,182	434.3
1953-1	3,893	11,711	423.2	14,415	11,823	421.8
1952-2	7,147	12,320	444.1	25,730	12,276	439.1
1952-1	3,304	11,806	423.4	12,798	11,851	419.7
1951-2	5,398	12,042	434.4	20,151	12,224	435.3
1951-1	2,494	11,584	417.2	10,513	11,711	416.2
1950-2	4,401	12,029	432.6	16,839	12,168	431.1

DISCUSSION

There are some assumptions which need to be fulfilled, for the contemporary comparison to provide unbiased estimates of the difference between AI and non-AI merit. An obvious one is that the dams of the AI and non-AI progeny have equal merit, or at least are randomly chosen to be mated to AI sires or to natural-service bulls. There is no reported evidence to the contrary. Beal and Madden (1) found no discrimination among dams for mating to AI or to non-AI sires, and also reported that many cows were mated in consecutive years by both methods. Tucker *et al.* (12) reported no significant difference among records of cows mated to AI sires or to natural-service bulls, although production of the cows mated to natural-service sires was higher than for cows mated to AI sires.

Another assumption is that the natural-service bulls in the herds where comparisons are available are a random sample of natural-service bulls. If the

non-AI sires in these herds are above the average of non-AI sires, then the contemporary comparison biases downward the estimate of superiority of AI progeny. Conversely, if the non-AI sires are below the average, then the AI superiority is actually less than the estimate would indicate. This assumption is needed only if inferences are to be made beyond the herds in which comparisons occur. To apply to the New York population of Holsteins, the assumption must hold.

Another assumption, required if inferences are to be extended beyond the herds which are on DHIA testing, is that the DHIA herds are a random sample of New York herds or at least make use of natural-service sires which can be considered to be drawn randomly from the New York population of natural-service sires.

The estimates of AI superiority will also estimate the trend in AI genetic merit, if it can be assumed that the genetic merit of the non-AI progeny has remained constant over the time period under study, a rather unlikely possibility.

The pattern of relatively great superiority of AI over non-AI progeny in 1951, followed by a decline in 1952 and 1953, is similar to that described by Elston (3) for an analysis of some of the same records. Elston relied on the assumption that various biases cancelled each other, in order to predict trend in genetic merit of AI sires by the method of least-squares. His study included progeny of 54 AI Holstein sires calving between 22 and 42 mo. of age in the period between March, 1949, and February, 1957, inclusive. The results presented in Table 2 also agree in general with those of Elston (3), in that the trends are upward in 1954 and 1955 but do not agree in 1956, the last year of the Elston study, when he found the greatest gain in genetic value. In the present study, a slight decline occurred after 1955 until 1959. The superiority in 1959 is greater than in any other year included in the study.

If the Dean bull had been excluded from the analysis, the AI progeny of 1951 through 1953 would have had little if any advantage over their non-AI contemporaries. The decline of production in 1952 and 1953 probably was caused by the fact that the Dean daughters made up a smaller proportion of total AI progeny in 1952 and 1953 than in 1951. During the corresponding breeding period, practically all sires purchased were used in the permanent breeding stud. (Most of the AI progeny in New York can be attributed to one cooperative artificial breeding organization, particularly in the earlier years.) The gain after 1953 corresponds to the period during which some of these sires could be eliminated because of better methods of extending semen and because more bulls were available, and after evaluation of the first AI progeny records of some of the earlier sires. From 1954 through 1958, a plateau of superiority was evidently reached. The slight decline from 1956 to 1958 may have been due to the increased demand for semen during the corresponding insemination period, resulting in the greater use of some bulls which had unknown genetic value except for knowledge of their natural-service daughters, Carter (2). The striking increase in 1959 may be accounted for by the initiation of a young sire sampling program by the cooperative artificial breeding organization in

1950 and 1951. Young sires first used at this time and then retired from service had a sufficient number of daughter records 4 or 5 yr. later for evaluation of these bulls. The better bulls were selected for assignment to the permanent breeding stud. The influence of the selected sires on the population would begin to appear after another 4 or 5 yr. or, in this case, in 1959. The increased number of evaluated sires available also allowed for culling of some members of the permanent stud possessing low ratings. The young sire program has been continued, although certainly not on a large scale when compared to the project undertaken in New Zealand, reported by Searle (10). The effect on the 1959 production does, however, indicate that progress can be made by young sire sampling, together with rigorous culling of bulls on the basis of their daughter records consistent with the semen needs of the stud.

For many years sires have been evaluated largely on the basis of fat production of their progeny with less regard to milk production. No doubt this emphasis will be changed in the future. The emphasis on fat records does show up in the trend of superiority. The fluctuations by years are proportionately less for fat than for milk superiority. And relatively more progress has been made toward increasing the fat superiority of AI progeny over non-AI contemporaries. Increased weight to milk production relative to fat production should allow for larger gains in the milk advantage of AI cows than have occurred since 1951.

Comparison of Tables 2 and 4 shows that errors in estimating AI superiority would occur if over-all averages of AI and non-AI animals were used. If over-all averages were used, the AI progeny would appear to have been poorer than non-AI progeny during 1950 to 1953 for milk production and only slightly better for fat production. In the period 1956 through 1959 the superiority of the AI progeny would have been overestimated. A possible explanation for the low over-all AI average in the early 1950's is that most of these animals were probably young and unselected, whereas the non-AI cows were of all ages. The older cows would undoubtedly have been a selected group. There probably would have been also a tendency among the better herds to use their own bulls until AI was proven. This would have adversely weighted the herd effects against the AI progeny average.

There seems to be no ready explanation for the bias in favor of AI in the later years. One possible reason is that the progeny averages are published for bulls in the breeding stud. Culling of cows may be done on this basis. Similar information would not be available for non-AI sires. Or, perhaps the balance has swung to where the better herds are using AI exclusively.

CONCLUSIONS

The genetic merit of AI progeny for both milk and fat production was superior to contemporary non-AI progeny for all years of calving from 1951 through 1959.

Superiority for fat production was more evident than for milk production, indicating that selection emphasis on fat records of progeny was greater than

that on milk records. Superiority for milk production could be increased more rapidly if selection of sires was based on progeny milk records rather than fat records.

The gain in genetic merit has been about one-half per cent per year since 1953 until 1959, if it can be assumed that merit of non-AI progeny has remained constant. More young bulls should be sampled, so that increased selection intensity may be employed which would result in greater superiority of AI progeny over non-AI progeny.

Biases in estimating superiority of AI progeny over non-AI progeny will result if over-all averages are used rather than within-herd-year-season contemporary comparisons of unselected progeny.

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USE OF BIMONTHLY RECORDS IN HERD TESTING

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SUMMARY

Records calculated from herd-testing figures obtained in alternate months (bimonthly records) were compared with those calculated from monthly herd testing, in a study involving 35 herds. Comparison of herd averages, within-herd ranking of cows, and repeatability of records under the two systems indicated that bimonthly records are suitable for within-herd comparisons but not for between-herd comparisons. The within-herd ranking of cows was almost identical and the repeatability of the bimonthly records was only 0.04 lower than that of the monthly records. Sire proofs calculated from the two sets of records in these herds ranked the bulls similarly.

The possibility of introducing bimonthly herd-testing was considered in New Zealand in 1955, following a sharp decline in the number of cows tested. It was thought that the cost of testing under the monthly system—the only system then in operation—had contributed to this decline as costs had doubled since the war. It became important, therefore, to examine the efficiency of cheaper systems of testing, particularly one whereby cows would be tested in alternate months instead of every month, as this could easily be incorporated with the existing system. This paper reports the investigations which led to bimonthly testing being recommended and presents some of the effects of its being adopted.

As early as 1927 it was reported by McDowell (9) that bimonthly records were on average 3.8% less accurate than daily records, whereas monthly records were 2.9% less accurate. Gifford (5), in 1930, reported correlation coefficients greater than 0.95 between bimonthly and total records, and McKellip and Seath (10) found correlations of 0.97 to 0.99 between monthly and bimonthly records. More recently, several workers (1, 2, 3, 8) have made comparisons between daily, monthly, bimonthly, and quarterly recording, in terms of percentage errors of the one system against another. Generally speaking, their conclusions have been as stated by Bayley *et al.* (2), that "the frequency of the large errors indicates that bimonthly records should be satisfactory for sire proving and population studies, but they may be unsatisfactory when used as individual lactation records."

The main uses of dairy records are for culling low-producing cows from the herd, for selecting replacement calves from the top-yielding cows in the herd and for sire proving. Records are used on a within-herd basis for these purposes and also for most genetic and analytical studies. Evaluation of bimonthly testing as an alternative to monthly testing was, therefore, made in terms of its efficiency for within-herd comparisons, rather than its efficiency for estimating total lactation yield per se. Three comparisons of the two systems of testing were made.

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Firstly, herd averages were compared to examine the effect on between-herd comparisons of the particular set of alternate months in which bimonthly tests would be made. Secondly, the value of bimonthly testing records as a basis for culling was examined by comparing the ranking of cows according to their records calculated from bimonthly tests with their ranking on records calculated from monthly tests. Thirdly, the value of bimonthly testing for predicting future production was compared with that of monthly testing by a comparison of repeatability estimates.

DATA

The monthly production records for all cows in 35 herds were available for four dairying years, 1951-1952 to 1954-1955. All herds were used in the comparison of herd averages, but only 30, which were predominantly Jersey (New Zealand's main breed) were used for the repeatability and ranking comparisons. The same data were used by Castle and Searle (4) in studying the repeatability of monthly records. All studies were made on milk fat, as this is the economically important product of New Zealand's dairy industry.

The pattern of dairy farming in New Zealand is seasonal, with most cows calving in August and September and many herds drying off before the end of May, so that few herds are tested in June and July. As the herd-testing year starts in July, with bimonthly testing herds could have their first test either in July or in August, with the opportunity of six tests, although in practice the majority would not start until August or September and have five tests, or some only four. Lactation yields calculated from bimonthly testing were, therefore, obtained in two ways, using the records for every other month starting in August and for every other month starting in September. The lactation yields based on monthly testing (which we shall refer to simply as monthly records) were calculated from the same test-day records, in the normal way by centering the testing date and calculating production in the first period from four days after calving to 15 days after the first test day, with 30-day periods thereafter. In this way, the lactation is calculated up to approximately 15 days after the last test; drying-off dates are not recorded in New Zealand. The lactation yields calculated from bimonthly testing (which we shall call bimonthly records) were obtained in each case by multiplying the average of the test-day yields over the appropriate alternate months, by the number of days in the period covered by the monthly record. The bimonthly records were, therefore, calculated in a manner slightly different from what would be used in practice, as lactation length would then be measured up to approximately 30 days after the last bimonthly test.

The average fat yields according to age for the 30 Jersey herds are shown in Table 1, age being taken to the nearest year. The averages for the bimonthly records are close to those of the monthly records, and accordingly all records were age-corrected by the multiplicative gross comparison factors derived from the monthly records, the 5- to 9-yr.-olds being regarded as mature cows. These are the factors given in (4).

TABLE 1

Average fat yield (pounds) according to age

Age (*years*)	No. of cows	Method of testing		
			Bimonthly	
		Monthly	Starting August	Starting September
2	1,165	261	261	259
3	990	297	299	292
4	852	328	328	324
5–9	2,550	350	350	353

COMPARISON OF HERD AVERAGES

The average uncorrected milk fat yield per cow in each herd was calculated each year for the three sets of records, and comparison made on a within herd and year basis. Table 2 shows the distribution of the bimonthly herd averages

TABLE 2

Distribution of bimonthly herd averages in relation to monthly herd averages

Bimonthly average in relation to monthly average	Bimonthly testing	
	Starting August	Starting September
	No. of herds	
Larger	83	31
Smaller	52	97
Equal	5	12
35 herds, 4 yr.	140	140

in relation to the monthly averages, as to whether larger, smaller, or equal. There is a tendency for the bimonthly average, where testing starts in August, to exceed the monthly average (59% of 140 comparisons), and where testing starts in September, to be less than the monthly average (69% of comparisons). This result, on a herd basis, corresponds to that of Bayley *et al.* (2), August approximating to the first month of lactation.

Table 3 shows the distribution of the differences considered in Table 2, expressed as percentages of the monthly average, disregarding the sign of the

TABLE 3

Distribution of differences (without sign) between monthly and bimonthly herd averages (35 herds in 4 yr.)

Difference as per cent of monthly average	Percentage distribution of herds
Less than 2.5	64
2.5–4.9	31
5.0–9.9	5
10.0 and over	....

differences. In 64% of the cases the bimonthly average was within 2.5% of the monthly average, and in 95% of the cases it was within 5%. An implication from these results might be that bimonthly records are as suitable for between-herd comparisons as monthly records. However, if used for this purpose, differences between herd averages could include differences between the two bimonthly testing systems (starting in August and starting in September). A comparison between the two possible bimonthly herd averages for each herd was, therefore, made, as shown in Table 4.

TABLE 4

Distribution of differences (without sign) between August-starting and September-starting bimonthly herd averages (35 herds in 4 yr.)

Difference as per cent of monthly average	Percentage distribution of herds
Less than 2.5	33
2.5–4.9	36
5.0–9.9	28
10.0 and over	3
Total	100

In only one-third of the herds was the difference between the August-starting bimonthly average and the September-starting bimonthly average less than 2.5% of the monthly average; in approximately one-third of the herds the bimonthly averages differed by 5% or more of the monthly average. The largest percentage difference recorded for a herd was 16.5%; the monthly average was 254 lb. milk fat, whereas the August-starting bimonthly average was 279 lb. and the September-starting average was 237 lb. Differences as large as these, together with the bias seen in Table 2, invalidate any suggestion of using bimonthly records for between-herd comparisons, unless some correction is made according to month of first test.

RANKING OF COWS WITHIN HERDS

Farmers usually cull some of the lowest-producing cows from their herds each year. The culling is made not so much on the exact measure of a cow's production as on her relative position in the herd. Bimonthly records would be as suitable as monthly records for this purpose if the order in which the cows of a herd are arrayed, from the highest produced to the lowest, were essentially the same for the two kinds of records. To test this possibility a rank correlation coefficient, that is, the correlation between the array of the cows according to their monthly records and the array of the same cows according to their bimonthly records in the same year, was calculated for each of 30 herds. Age-corrected records were used and the correlation was calculated for each herd in each of 3 yr., using monthly records and bimonthly records starting in August. The coefficient used was Spearman's ρ, as given in (7). The distribution of values obtained is shown in Table 5. All values exceeded 0.81, and 70% of them

TABLE 5

Distribution of rank correlation coefficients for the cows of 30 herds arrayed according to their monthly records and bimonthly records starting in August

Rank correlation coefficient	No. of herds		
	1951–1952	1952–1953	1953–1954
.96 to 1.00	11	10	7
.91 to .95	15	16	15
.86 to .90	3	3	4
.81 to .85	1	1	4
Total	30	30	30

exceeded 0.90. All are significantly greater than zero at the 0.1% level and consistently close to 1.00. No coefficients were calculated for other seasons or for bimonthly records starting in September, as the rankings for these closely resembled those for which coefficients had been calculated. The conclusion is that the cows within a herd are ranked similarly by bimonthly records and by monthly records. The culling of low producers on bimonthly records, therefore, removes from the herd almost exactly the same cows as would be removed by culling on monthly records, so that bimonthly records are considered satisfactory for culling within a herd.

REPEATABILITY

Repeatability estimates were obtained for the bimonthly records, using the same analyses as those reported by Castle and Searle (4), the data being from the 30 Jersey-predominant herds. These consisted of 5,557 lactations from 2,436 cows over 4 yr. Records were calculated for both August-starting and September-starting bimonthly testing, but repeatability was not estimated for these two groups as such. It was felt that if bimonthly testing were introduced, herds starting in August one year might start in September the next year. The data were accordingly analyzed by taking half the herds as starting in August the first year, September the next and so on, and the other half as starting in September the first year, August the second year and so on, over the 4 yr. These comprised one grouping of the records, the pattern of first months being reversed to form a second grouping. Table 6 sets out the division of the herds for the two groups.

TABLE 6

Grouping of bimonthly records for repeatability analysis

Herd codes	First month of testing, over the 4 yr. (A = August; S = September)	
	Group 1	Group 2
1–15	A, S, A, S	S, A, S, A
16–30	S, A, S, A	A, S, A, S

The repeatability estimates are shown in Table 7, alongside those for the monthly records taken from Castle and Searle (4). The estimates for bimonthly

TABLE 7

Within-herd repeatability estimates

Analysis	Monthly records [a]	Bimonthly records	
		Group 1	Group 2
Years random	.49	.44	.44
Years eliminated	.61	.55	.59

[a] Taken from Castle and Searle (4).

records are less than for monthly records, but only to an extent of about 10% of the values for monthly records.

The two analyses shown in Table 7 differ in their treatment of year effects. These are treated as random variables in the years-random analysis and as fixed effects in the years-eliminated analysis, in which they are eliminated from the variance components estimates by the use of Henderson's Method 3 (6). The difference between these analyses observed in the monthly records (4) also appears in the bimonthly records, the repeatability estimates when years are treated as fixed effects and eliminated being approximately one and a quarter times the estimates obtained in the years-random analysis. The variance components used in obtaining these repeatability estimates are given in (4) for the monthly records; the error components in the bimonthly records are approximately 20% greater than those in the monthly records, and the total variance of bimonthly records is approximately 10% higher than that of monthly records.

The slightly lower repeatability values for bimonthly records compared with monthly records can be expressed in terms of relative efficiency for prediction purposes. Taking 0.57 as the pooled estimate for the bimonthly records in the years-eliminated analysis, the value of bimonthly records for predicting productive ability from a single record can be expressed as $\sqrt{0.57/0.61} = 96.6\%$ of the value of monthly records for this purpose. The comparable value in the years-random analysis is $\sqrt{0.44/0.49} = 94.8\%$. Both cases represent only a small loss of efficiency if bimonthly records are used for predicting a cow's production ability from a single record.

OPERATION OF BIMONTHLY TESTING

Bimonthly testing, or alternate-month testing (A.M.T.) as it is called in New Zealand, was introduced in this country in the 1957-1958 dairy season. The number of herds tested under the bimonthly system is shown in Table 8. The fee for bimonthly testing in its first year of operation was two-thirds of

TABLE 8

Herds testing under the bimonthly system

Year	No. of herds	Per cent of all tested herds
1957–1958	920	12
1958–1959	1,015	15
1959–1960	1,007	15

that for monthly testing and this has since been reduced to 60% of the monthly testing charge in most parts of the country. It may seem surprising, therefore, that more herds have not changed from the monthly to the bimonthly system. One reason is that approximately 40% of tested herds contain pedigree cows, which precludes their adopting bimonthly testing if official pedigree records are desired, since the pedigree breed societies do not recognize bimonthly records. Also, the desire for regular monthly test returns probably deters many farmers from changing to the less expensive bimonthly testing.

Farmers adopting bimonthly testing in its first year of operation, 1957-1958, seemed favorably inclined towards it. A postal survey among the 920 farmers yielded returns from 417. Of these, 88% classified their general reaction as good or fair and the remainder classified bimonthly testing as poor. Of the farmers using bimonthly testing in 1957-1958, but previously testing under the monthly system, 59% continued testing after 1957-1958. As owners of 60% of the newly tested herds also continued testing, it appears that farmers using the bimonthly system and coming into testing for the first time are just as likely to continue testing as those who tested previously under the monthly system.

SIRE PROOFS ON BIMONTHLY RECORDS

Seven sires in natural service in the herds used for the repeatability study had six or more daughters each. Progeny tests calculated from these daughters on both the monthly and bimonthly records are shown in Table 9, the sire rating

TABLE 9

Sire proofs of bulls used in natural service, based on monthly and bimonthly records of the same daughters

		Rating		
			Bimonthly records	
Sire	No. of daughters	Monthly records	Starting August	Starting September
A	19	− 5	− 7	0
B	9	+31	+21	+32
C	8	+23	+20	+22
D	12	−12	−13	−11
E	13	− 4	− 4	0
F	19	+ 5	+ 1	+ 9
G	13	+ 2	− 2	− 3
Mean	13	+ 6	+ 2	+ 7

being the estimated superiority of the bull's daughters over daughters of the average bull. Generally speaking, the three sets of proofs are similar, Bulls B, C, and F being the highest ranked on each set of records. The proofs based on August-starting bimonthly records were, on average, 4 lb. milk fat lower than those based on monthly records; whereas, those based on September-starting bimonthly records were 1 lb. milk fat higher. These differences must be considered extreme, because in practice proofs would be based on more than 1 yr.'s

records and, since herds would not start testing in the same month each year, the differences between proofs based on monthly and bimonthly records would be less than those shown here.

Eleven sires used in artificial breeding now have some daughters tested under the monthly system and others under the bimonthly system. Sire proofs from both groups of daughters are shown in Table 10. The average daughter

TABLE 10

Sire proofs of bulls used in artificial breeding based on daughters with monthly records and others with bimonthly records

Sire	Proof based on monthly records		Proof based on bimonthly records	
	No. of daughters	Rating	No. of daughters	Rating
1	641	+16	142	+14
2	394	+ 2	94	+ 3
3	700	+50	121	+40
4	494	− 9	94	− 5
5	271	+ 9	58	+ 6
6	258	+19	77	+23
7	652	+27	131	+25
8	361	+39	93	+31
9	376	+27	88	+21
10	249	+49	74	+37
11	353	+12	62	+ 6
Mean	432	+22	94	+18

superiority of the 11 sires is estimated from the monthly records as 22 lb. milk fat on an average of 432 daughters per sire. The estimated mean superiority from the bimonthly records is 18 lb. milk fat from an average of 94 daughters per sire. The ranking of the bulls is, however, the same in both cases. These results support the conclusion that bimonthly records are, for practical purposes, as accurate as monthly records for sire-proving based on within-herd comparisons.

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GROWTH AND PRODUCTION CHARACTERISTICS OF HOLSTEIN-FRIESIAN, BROWN SWISS, AND RED SINDHI CROSSBRED FEMALES IN LOUISIANA AND MARYLAND [1]

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SUMMARY

Red Sindhis were crossed with Holstein-Friesians (H) at two stations and with Brown Swiss (BS) at a third station for the purposes of studying growth and productive adaptability of dairy cattle in the southern region of the United States. The growth and productive performances of 33 F_1 and 21¾ H animals have been compared with their purebred H contemporaries or herd-mates. Productive performances of 30 F_1 and 25¾ BS have been compared with their BS herd-mates.

The F_1 and ¾ H animals were significantly smaller ($P = <0.01$) at birth, and 3, 6, 12, and 18 mo. of age, and at 90 days postpartum than their H contemporaries. Growth rates of these two crossbred groups were not significantly different from each other.

All of the crossbred groups were significantly ($P = <0.01$) lower in average milk and fat yield during the first lactation than their H or BS herd-mates or contemporaries, with the exception of the fat yield difference between the ¾ BS and their BS herd-mates, which was not significant. Differences between the F_1's and their European contemporaries were greater ($P = <0.05$) during the summer than in the winter season at the two Southern stations. These results did not indicate superior productive adaptability of the crossbreds to a hot, humid climate.

The studies and observations of Bonsma (1-3) and Rhoad (15-17), of the genetic differences in animal response to climatic stress, stimulated considerable research on cross-breeding beef cattle of European origin with cattle of the several breeds of India. As pointed out by Rice *et al.* (18) and Shrode (21), the continuous cross-breeding of beef cattle for the production of commercial animals for slaughter purposes has met with considerable success in the South. In view of these results, the Red Sindhi, which is one of the breeds of Zebu cattle found in India that has a higher inherent milk productivity than is characteristic of the Zebu as a whole, was introduced into this country in 1946 for the purposes of studying genetic and physiological mechanisms of adaptability of dairy cattle in the Gulf Coast region. Reports on the growth and development (10, 12) and on the production characteristics (11) of Red Sindhi–Jersey crossbreds in Georgia, Louisiana, and Maryland have already been made. Also, several papers (5, 13, 19, 20) on the physiological aspects of heat tolerance or adaptability have been published.

The purpose of this paper is to report on the growth and production characteristics of various combinations of Red Sindhi and Holstein-Friesians in

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Louisiana and Maryland and on the production characteristics of various combinations of Red Sindhi and Brown Swiss in Louisiana, as compared to their purebred Holstein-Friesian and Brown Swiss stable-mates or contemporaries at the respective stations. A preliminary report on the Red Sindhi–Holstein cross-breeding has already been made (4).

EXPERIMENTAL PROCEDURES

Breeding plan. The breeding plan at each of the three stations was to produce F_1 and back-cross animals (¾ European–¼ Sindhi). In this paper the stations will be referred to as Baton Rouge, Beltsville, and Homer.

Two purebred Red Sindhi bulls were mated to representative Holstein-Friesians in each of the Baton Rouge and Beltsville herds to produce the F_1 crosses (½ Holstein–½ Sindhi). Red Sindhi bulls S-51 and S-53 were used at Baton Rouge, and Bulls S-51 and S-52 were used at Beltsville. One of the animals classified as a back-cross at Beltsville was sired by an F_1 bull, and the dam was a purebred Holstein cow. All other ¾ Holstein–¼ Sindhi animals at Beltsville were produced by mating five purebred Holstein-Friesian bulls to F_1 cows. Only one purebred Holstein-Friesian bull was used at Baton Rouge to produce this breeding group. In this paper, the first-generation crossbred group (½ Holstein–½ Sindhi) and the back-cross group (¾ Holstein–¼ Sindhi) will be referred to as F_1 and ¾ H, respectively. Although the ¾ H females were mated to Holstein bulls, only a few of the resulting offspring (⅞ H) were permitted to reach breeding age before being culled from the herds.

The procedures to produce the ½ Brown Swiss–½ Sindhi (F_1) and the ¾ Brown Swiss–¼ Sindhi (¾ BS) at the Homer station were similar to those outlined above. Two purebred Red Sindhi bulls, S-51 and S-53, were mated to representative purebred and high-grade Brown Swiss females to produce the F_1 group. Three purebred Brown Swiss bulls were mated to the F_1 females to produce the ¾ BS group.

Matings were made to keep inbreeding at a minimum. All crosses had less than 4.0% inbreeding.

Feeding and management. The within-herd feeding and management practices were similar for the crosses and the Holstein-Friesian or Brown Swiss stable-mates. The feeding program at Beltsville was superior to that of the Baton Rouge or Homer station, due to the fact that dry-lot feeding of alfalfa hay, corn silage, and grain was practiced throughout the year at this station. Both temporary and permanent pastures were used at the Baton Rouge and Homer stations for several months of each year. Quality of these pastures was rather poor during the late summer and early fall months. Both alfalfa and grass hay of average to poor quality was fed along with oat, corn, or grass silage of average to poor quality during late fall and winter months at Baton Rouge. Excellent grazing of clover and grass pasture was available during the spring months at this station. All hay fed at the Homer station was either oat or Coastal Bermuda grass, with the latter predominating. The silage fed at this station was either oat or millet. The quality of the feeding and management practices declined from 1955 to 1958 at the Homer station.

The heifers in each of the breed groups were bred at the first estrus after 15 mo. of age. The cows were bred 60 to 90 days postpartum. All cows were machine-milked twice daily at each of the three stations. All crossbreds were given an opportunity to complete at least one record, but in subsequent lactations the culling rate was high. The proportion of crossbred animals culled from the herds was higher than that for the purebreds, due to the very low production of the crossbreds in many instances. Grain feeding was done either just prior to or during each milking. The level of grain feeding was approximately 1:4 (1 lb. grain for each 4 lb. 4% FCM). Body weights of the animals were taken at birth and at 3, 6, 12, and 18 mo. of age as well at at 90 days postpartum at Baton Rouge and Beltsville. This also was done at the Homer station, but these data will not be reported in this paper.

Analyses of data. In this study the crossbreds were compared to their purebred European contemporaries or stable-mates. The contemporaries were the first-lactation European animals calving the same year and season in the same herd as the crossbreds. The stable-mates were all Holstein-Friesians or Brown Swiss calving the same year and season in the same herd as the crossbreds.

The 2×, 305-day (or less) milk and fat production records of all animals were grouped into seasons by date of calving (April–September or October–March). The individual Holstein-Friesian and Brown Swiss records were adjusted to a mature equivalent (M.E.) basis by using the DHIA age-correction factors for the respective breeds. Then year-season Holstein-Friesian and Brown Swiss means were adjusted to the age of calving for each crossbred in the corresponding year-season at each station. The deviation for each crossbred from the Holstein-Friesian or Brown Swiss stable-mate average was determined. Since the numbers of Holstein-Friesians or Brown Swiss were small in some year-season-station groups, the observed differences between the crossbred record and year-season-station age-adjusted record was weighted by the factor $\frac{n}{n+1}$. This procedure was considered more satisfactory than attempting to adjust the crossbred records to a M.E. basis, since no satisfactory or reliable age-correction factors are available for use on the crossbred records.

In addition to comparing the F_1's with their contemporaries and stable-mates as outlined above, they were compared with their purebred European maternal half-sisters, using first lactations only. Also, ¾ H and ¾ BS females were compared with contemporaries, as well as with their contemporary purebred paternal half-sisters.

To provide tests of significance, the same general statistical procedures as outlined by McDowell *et al.* (11) were used. This was done in order to evaluate the importance of some of the sources of variation in the weighted differences described above. Two least-squares analyses were completed. In the first analysis, the effect of length of lactation was not removed statistically. In the second analysis, the mathematical model was extended in order to remove statistically, by linear and quadratic regression, the effect of length of lactation.

RESULTS AND DISCUSSION

Growth data for crossbreds and Holsteins. Table 1 shows the average body weights of the F_1, ¾ H, and Holstein-Friesian females at the various ages at Baton Rouge and Beltsville. The contemporary Holstein-Friesian females were significantly ($P = < 0.01$) larger animals than the females in either of the crossbred groups at each of the various ages. However, the two crossbred groups did not differ significantly from each other. Station differences were highly significant ($P = < 0.01$). Nevertheless, the trends of growth rates were similar for each of the breed groups at each station. Differences in growth rates for each of the breeding groups at the two stations were probably due to differences in the levels of feeding, parasites, and effects in climate. The major difference in the feeding programs was in the quality of forages fed.

TABLE 1

Average body weights (in pounds) of the Holstein-Sindhi crossbred and contemporary purebred Holstein females at various ages

Breeding group	Station	No. of animals	Birth	3 mo.	6 mo.	12 mo.	18 mo.	90 Days post-partum[b]
F_1	Beltsville	13	80	200	377	672	799	1,036
	Baton Rouge	20	75	157	292	524	683	840
	Both	33	77	174	325	582	729	917
¾ H	Beltsville	8	70	196	359	618	793	999
	Baton Rouge	13	75	159	291	517	674	883
	Both	21	73	174	319	558	723	930
Holstein[a]	Beltsville	....	90	221	421	765	971	1,228
	Baton Rouge	....	81	173	307	516	690	969
	Both	....	86	197	364	640	830	1,098

[a] All contemporary Holsteins in each of the two herds.
[b] Body weight 90 days after first calving.

These results on growth rate are not in agreement with those reported by McDowell (10, 12) and Rathore (14). McDowell *et al.* (12) reported that, in general, the Sindhi-Jersey crossbreds gained faster than Jerseys up to 12 mo. of age, but only the F_1 and the ⅝ J crosses remained larger at 18 mo. of age and at the first lactation. Rathore (14) observed that F_1's showed superiority in size and growth rate to either Jersey or the Red Sindhi cattle. In the studies reported herein the F_1's were smaller than the Holsteins at birth and at all ages through first calving. In interpreting these conflicting results, it must be remembered that the Holstein-Friesian female is on the average a much larger animal at birth and grows much more rapidly than does the Jersey or the Red Sindhi female. Also, it must be noted in Table 1 that the numbers of crossbred animals involved in these studies were small.

The results for the ¾ Holstein females are difficult to explain, since their growth rates were almost identical with those of the F_1's. Sampling errors due to small numbers of animals in the ¾ H group may have been a factor. Also, only one Holstein sire was used at Baton Rouge to sire the 13 ¾ H females.

Production data for crossbreds and Holsteins. Tables 2-5 present the production data on the crossbreds and the differences between their records and that of their Holstein contemporaries or maternal half-sisters or paternal half-sisters. Also, the crossbred average records are expressed as a percentage of Holstein records in Tables 2-4. It should be emphasized that the crossbreds showed tremendous ranges in production. The F_1's ranged from 306 to 9,804 lb. milk and from 13 to 535 lb. fat at the two stations. About one-third of these crosses were very low producers. This is reflected in the data presented in Table 5. The ¾ H group was also quite variable, ranging from 292 to 12,273 lb. milk and from 9 to 556 lb. fat.

Tests of significance among the least-squares means for main effects and interactions in the data from which the information shown in Table 2 was derived revealed that breed group, season of calving, and station differences

TABLE 2

Comparison of Holstein-Sindhi crossbreds with purebred Holstein contemporaries for first lactations only using DHIA age-correction factors for Holstein records

Breed group	Season of calving	No. of cows	Age at calving	Crossbred[a] records		Holstein[b] herd-mates records		Differences (crossbreds minus Holsteins)		Crossbred records as percentage of Holsteins	
				Milk	Fat	Milk	Fat	Milk	Fat	Milk	Fat
			(mo.)	*(lb.)*							
				Beltsville							
F_1	Oct.-Mar.	7	25	5,612	305	12,516	497	−6,904	−192	45	61
	Apr.-Sept.	6	26	5,977	312	12,484	507	−6,507	−195	48	62
	Both	13	26	5,780	308	12,501	502	−6,721	−194	46	61
				Baton Rouge							
F_1	Oct.-Mar.	8	28	5,120	225	7,704	259	−2,584	− 34	66	87
	Apr.-Sept.	12	30	3,277	141	7,793	270	−4,516	−129	42	52
	Both	20	29	4,014	175	7,758	266	−3,744	− 91	52	66
				Beltsville and Baton Rouge							
F_1	Oct.-Mar.	15	27	5,350	262	9,949	370	−4,599	−108	54	71
	Apr.-Sept.	18	29	4,177	198	9,357	349	−5,180	−151	45	57
	Both	33	28	4,710	227	9,626	359	−4,916	−132	49	63
				Beltsville							
¾ H	Oct.-Mar.	6	25	10,696	474	12,587	494	−1,891	− 20	85	96
	Apr.-Sept.	2	26	8,797	397	11,984	467	−3,187	− 70	73	85
	Both	8	25	10,221	454	12,436	487	−2,215	− 33	82	93
				Baton Rouge							
¾ H	Oct.-Mar.	5	32	4,429	160	8,049	283	−3,620	−123	55	57
	Apr.-Sept.	8	32	4,770	171	8,260	293	−3,490	−122	58	58
	Both	13	32	4,639	167	8,179	289	−3,540	−122	57	58
				Beltsville and Baton Rouge							
¾ H	Oct.-Mar.	11	28	7,847	331	10,524	398	−2,677	− 67	75	83
	Apr.-Sept.	10	31	5,576	216	9,005	328	−3,429	−112	62	66
	Both	21	30	6,765	276	9,801	365	−3,036	− 89	69	76

[a] Actual 2×, 305-day or less records.

[b] Holstein records were adjusted to the same age basis as the crossbreds, using DHIA factors for Holsteins.

were highly significant ($P = < 0.01$). Adjustment for length of lactation made little difference in either the least-squares means or the tests of significance for differences among breed groups, seasons of calving, stations, or interactions of these main effects. In this analysis of variance the length of lactation was held constant. It was found that none of the interactions was significant in either of the two analyses of variance conducted on these data.

Holstein offspring of Holstein cows produced significantly ($P = < 0.01$) more milk and fat than their maternal half-sisters sired by Red Sindhi males (Table 3). The Holstein maternal half-sibs were not significantly different from

TABLE 3

Comparison of Holstein-Sindhi crossbred cows (F_1) with their purebred Holstein maternal half-sisters using first lactations only

Season of calving	F_1				Holstein maternal half-sisters[b]			Differences (F_1's minus Holstein maternal half-sisters)		F_1's records as per cent of half-sisters	
	No. of cows	Age at calving	Actual[a] production		No. of cows						
			Milk	Fat		Milk	Fat	Milk	Fat	Milk	Fat
		(mo.)	*(lb.)*	*(lb.)*		*(lb.)*	*(lb.)*	*(lb.)*	*(lb.)*		
					Beltsville						
Oct.-Mar.	7	25	5,612	305	6	11,845	475	−6,233	−170	47	64
Apr.-Sept.	6	26	5,977	312	6	11,081	459	−5,104	−147	54	68
Both	13	26	5,780	308	12	11,631	467	−5,851	−159	50	66
					Baton Rouge						
Oct.-Mar.	8	28	5,120	225	13	7,580	274	−2,460	− 49	68	82
Apr.-Sept.	12	30	3,277	141	6	8,842	300	−5,565	−159	37	47
Both	20	29	4,041	175	19	8,065	285	−4,024	−110	50	61
					Beltsville and Baton Rouge						
Oct.-Mar.	15	27	5,350	262	19	8,890	336	−3,540	− 74	60	78
Apr.-Sept.	18	29	4,177	198	12	10,033	382	−5,856	−184	42	52
Both	33	28	4,710	227	31	9,385	356	−4,675	−129	50	64

[a] Actual 2×, 305-day or less records.

[b] Holstein DHIA age-correction factors used to correct records of Holstein maternal half-sisters to same age basis as that of the F_1's. The average shown is an unregressed mean.

their Holstein stable-mates in milk or fat yield at either of the two stations. Differences shown in Table 3 are similar to those in Table 2 for the F_1's as compared to their Holstein contemporaries.

Statistical analyses of the data from which those shown in Table 4 were derived showed that the Holstein paternal half-sibs of the ¾ H produced significantly ($P = < 0.01$) more milk and fat than did the ¾ H females. The same was true for differences between stations and seasons of calving. The production of the Holstein paternal half-sibs did not differ significantly from that of their Holstein stable-mates. It is interesting to note that the differences in production between the ¾ H group and their Holstein contemporaries (Table 2), and between the ¾ H group and their Holstein paternal half-sibs (Table 4), were of the same general magnitude.

TABLE 4

Comparison of Holstein-Sindhi crossbreds (¾ H) with their contemporary purebred Holstein paternal half-sisters using first lactations only

Season of calving	¾ H				Paternal Holstein half-sisters[b]			Differences		Crossbreds as percentage of Holsteins	
	No. of cows	Age at calving	Actual production		Average No.						
			Milk	Fat		Milk	Fat	Milk	Fat	Milk	Fat
		(mo.)	*(lb.)*	*(lb.)*		*(lb.)*	*(lb.)*	*(lb.)*	*(lb.)*		
Beltsville											
Oct.-Mar.	6	25	10,696	474	6	12,654	496	−1,958	− 22	85	96
Apr.-Sept	2	26	8,797	397	1	10,594	375	−1,797	+ 22	83	106
Both	8	25	10,221	454	7	12,360	479	−2,139	− 25	83	95
Baton Rouge											
Oct.-Mar.	5	32	4,429	160	4	8,270	271	−3,841	−111	54	59
Apr.-Sept.	8	32	4,770	171	3	7,657	273	−2,887	−102	62	63
Both	13	32	4,639	167	7	7,893	272	−3,254	−105	59	61
Beltsville and Baton Rouge											
Oct.-Mar.	11	28	7,847	331	10	10,661	394	−2,814	− 63	74	84
Apr.-Sept.	10	31	5,576	216	4	7,984	285	−2,408	− 69	70	76
Both	21	30	6,765	276	14	9,456	345	−2,691	− 69	72	80

[a] Actual 2×, 305-day or less records.

[b] Holstein DHIA age-correction factors used to correct the Holstein average to same age basis as that of the crossbreds. The average shown in each case is a contemporary first-lactation group mean. Records were not regressed.

The production of the purebred Red Sindhi averages about 2,320 lb. milk in first lactations, according to Mahadevan (8). Stonaker (22) reported an average of about 2,638 lb. milk and 129 lb. fat for this breed in the Gangetic Plains region of India. His findings are in agreement with those of Sundaresan (23), who gave an average of 2,600 lb. milk for first lactations for the Red Sindhi. These animals were quite variable in production, as was demonstrated by the coefficient of variation of 40% for milk yield in the first lactation, reported by Mahadevan (8). The average correlation of the yield in one lactation with that of the next has been found to be 0.41, and the heritability of milk yield was probably of the order of 0.20 for the Red Sindhi (8, 22). Considering these findings on average milk yield of the Red Sindhi, along with those on heritability of the same trait, the results reported herein are in line with expectations for crosses between the Red Sindhi and Holstein-Friesian. Also, these results are in agreement with those reported by Maule (9) for Friesian × Zebu crosses on the military farms in India, and with those of Sundaresan (23) for the same type of crosses at the Allahabad Agricultural Institute in India.

The results presented in Table 5 indicate that the relative production of the Holsteins and crosses changed very little in later lactations. Even with some selection, the F_1's produced only slightly more relative to their Holstein herdmates in second than in first lactations. This was also true in the third lactation. These results are in good agreement with those of McDowell *et al.* (11) for Jerseys and Red Sindhi × Jersey crosses. Thus, as pointed out by McDowell *et al.* (11), it would appear that the inferiority of the crosses in the first lacta-

tion was apparently not a maturity factor. Without doubt, temperament was at least partially responsible for the lower production of the crosses, especially in the F_1's. As was observed by McDowell *et al.* (11), in many instances the extremely low producers were highly temperamental. They were also slow eaters in the barn and on pasture. Although these factors may help explain some of the differences in relative production of the F_1's and the Holstein shown in Table 5, these factors certainly do not make them any more useful for dairy purposes in this country.

TABLE 5

Comparison of Holstein-Sindhi crossbreds (F_1) with purebred Holstein herd-mates in the Baton Rouge herd using all lactations for crossbreds and purebreds

Lactation No.	Crossbred records: No. of cows	Crossbred records: Age at calving	Crossbred records: Length of lactation	Crossbred records: Actual production: Milk	Crossbred records: Actual production: Fat	Purebred Holstein[a] records (herd-mates)[a]: Milk	Purebred Holstein[a] records (herd-mates)[a]: Fat	Differences: Milk	Differences: Fat	F_1's record as a percentage of herd-mates: Milk	F_1's record as a percentage of herd-mates: Fat
		(mo.)	*(days)*	*(lb.)*							
1	20	29	221	4,014	175	7,758	266	−3,744	− 91	52	66
2	14	42	230	5,649	245	9,743	338	−4,094	− 93	58	72
3	8	55	245	5,855	239	9,835	342	−3,980	−103	60	70
4	5	70	270	6,372	274	10,007	345	−3,635	− 71	54	59
5	2	79	296	8,353	325	10,514	364	−2,161	− 39	79	89

[a] Holstein DHIA age factors used to correct records of Holstein herd-mates to the same age basis as that of F_1's. The average shown is an unregressed mean.

Studies of the weather patterns by Johnston *et al.* (7) showed that the climatic conditions in the Baton Rouge area are among the most critical areas in the United States for the productive performance of dairy cattle. Also, physiological studies (5, 6, 13, 19, 20) involving respiratory, body temperature, and heat production responses of Red Sindhi × Jersey, Red Sindhi × Holstein crossbreds, and purebred Jerseys and Holsteins in Louisiana and Maryland have shown that the crossbreds are more adaptable to hot, humid climatic conditions. As will be observed in Tables 2, 3, and 5, the productive performance of the F_1 group averaged only slightly more relative to the Holsteins at Baton Rouge than at Beltsville. However, F_1 animals calving during the hotter season (April-September) at Baton Rouge produced less relative to the contemporary Holsteins than did those calving in the cooler season (October-March). These differences were statistically significant ($P = < 0.05$). Such differences were not apparent in the $\frac{3}{4}$ H group. These results indicate that dairy cattle breeders in the southern region of the United States should be more concerned with productive adaptability than with physiological adaptability, as measured by respiratory and body temperature responses of dairy animals to heat stress. This is in line with the suggestion by Shrode (21), that present efforts can best be directed toward the development and use of the most effective methods of selection on objective measures of performance with respect to economic traits.

Production data for crossbreds and Brown Swiss. Tables 6-8 give the productive comparisons between the Brown Swiss contemporaries, herd-mates, maternal half-sisters and paternal half-sisters, and the crossbred groups at the Homer station. In interpreting these results it should be emphasized again that the level of feeding and management in this herd improved from 1950 through 1954 and then declined rather severely during the period 1955-1958. Consequently, differences in production between crossbreds and Brown Swiss were not as marked as they probably would have been under good feeding and management conditions. This is particularly true for the comparisons between the ¾ BS and Brown Swiss groups.

TABLE 6

Comparison of Brown Swiss-Sindhi crossbreds with contemporary Brown Swiss herd-mates

Breed group	Season of calving	No. of cows	Age at calving	Crossbred records[a]		Brown Swiss records[b]		Differences		Crossbreds as percentage of Brown Swiss	
				Milk	Fat	Milk	Fat	Milk	Fat	Milk	Fat
			(mo.)	*(lb.)*							
F_1	Oct.-Mar.	16	30	3,833	189	6,238	262	−2,495	−73	61	72
	Apr.-Sept.	14	29	2,661	129	4,810	196	−2,149	−67	55	66
	Both	30	30	3,286	161	5,620	231	−2,334	−70	58	70
¾ BS	Oct.-Mar.	11	30	5,093	216	5,835	239	− 742	−23	87	90
	Apr.-Sept.	14	32	5,012	231	5,408	217	− 396	+14	93	106
	Both	25	31	5,047	225	5,586	226	− 539	− 1	90	100

[a] Actual 2×, 305-day or less records.
[b] All ages used and converted to same age basis as crossbreds, using DHIA Brown Swiss age-correction factors.

Statistical analyses of the data from which those shown in Table 6 were derived revealed that the main effects, breed groups and season of calving, were highly significant ($P = < 0.01$) for milk yield. Differences in fat yield between the ¾ BS and Brown Swiss were not significant. Perhaps the feeding program as mentioned above had some effect on this comparison. The Brown Swiss maternal half-sisters of the F_1's produced significantly ($P = < 0.01$) more milk and fat than did the F_1's (Table 7). None of the differences shown in Table 8 were significant, except season of calving.

The relative production of the F_1 group to the Brown Swiss calving during the hotter season is less than that for animals calving during the cooler season, as shown in Tables 6 and 7. These results are in good agreement with those discussed previously for the Holsteins and Sindhi-Holstein F_1's at Baton Rouge.

General discussion. From the results of these studies, and the knowledge of the variation in productive ability and heritability of milk and fat yield that exists within and among the European breeds of dairy cattle, it would appear that research on systems of breeding other than crossing with the Red Sindhi would offer greater promise for developing dairy cattle for adaptability to con-

TABLE 7

Comparison of Brown Swiss–Sindhi crossbreds (F_1) with their Brown Swiss maternal half-sisters using first lactations only

Season of calving	F_1's[a]				Maternal half-sisters[b]			Differences		Crossbreds as percentage of maternal half-sisters	
	No. of cows	Age at calving	Actual production		No. of cows						
			Milk	Fat		Milk	Fat	Milk	Fat	Milk	Fat
		(mo.)	*(lb.)*	*(lb.)*		*(lb.)*	*(lb.)*	*(lb.)*	*(lb.)*		
Oct.-Mar.	16	30	3,833	189	14	6,731	256	−2,898	−67	57	74
Apr.-Sept.	14	29	2,661	129	4	5,316	212	−2,655	−83	50	61
Both	30	30	3,286	161	18	6,417	246	−3,131	−85	51	65

[a] Actual 2×, 305-day or less records.
[b] Brown Swiss DHIA age-correction factors used to convert records of Brown Swiss maternal half-sisters to same age basis as crossbred.

TABLE 8

Comparison of Brown Swiss–Sindhi crossbreds (¾ BS) with their contemporary paternal half-sisters

Season of calving	¾ BS[a]				Paternal Brown Swiss half-sisters[b]		Differences		All Brown Swiss herd-mates of paternal half-sisters[c]	
	No. of cows	Age at calving	Actual production							
			Milk	Fat	Milk	Fat	Milk	Fat	Milk	Fat
		(mo.)	*(lb.)*		*(lb.)*		*(lb.)*		*(lb.)*	
Oct.-Mar.	11	30	5,093	216	5,647	236	−554	−20	5,835	239
Apr.-Sept.	14	32	5,012	231	4,710	201	+302	+30	5,408	217
Both	25	31	5,047	225	5,136	217	− 89	+ 8	5,586	226

[a] Actual 2×, 305-day or less for first lactations.
[b] First lactations of contemporary Brown Swiss paternal half-sisters converted to same age basis as crossbreds using DHIA age-correction factors.
[c] All records of Brown Swiss herd-mates of paternal half-sisters converted to same age basis as crossbreds.

ditions in the southern region of the United States. This is the same conclusion reached by McDowell *et al.* (11) in their Red Sindhi–Jersey cross-breeding studies. The results reported herein and those reported earlier by McDowell *et al.* (11) indicate that it would take several generations of selection for productive performance within the F_1 groups to produce dairy cattle as productive as are the average European dairy cattle in the southern region of the United States at present. Also, the results reported by McDowell *et al.* (11) and Johnston (5) strengthen the contentions of many dairy cattle breeders that breeders should be more concerned with selection for productive performance within a European breed and with feeding high-quality forages throughout the year than with the introduction of Zebu breeding into their program.

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HYDROLYSIS, EXTRACTION, AND FRACTIONATION OF THE BILIARY ANDROGENS IN THE FEMALE BOVINE [1]

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SUMMARY

A systematic procedure has been developed for the hydrolysis, extraction, and fractionation of the androgens in the bile of the female bovine. The procedure described includes the following methods: (a) hydrolysis of the bile; (b) extraction of the hydrolysate; (c) separation of the ether-soluble material into acidic and neutral fractions; (d) separation of the neutral fraction into the alcoholic and nonalcoholic fractions; (e) separation of the neutral alcoholic fraction into the ketonic and nonketonic fractions; and (f) digitonin separation of the neutral hydroxyketones into alpha and beta fractions.

The fractionation procedure identified a digitonin-precipitable and a digitonin-nonprecipitable hydroxyketonic androgen from the neutral, saponifiable material of bile from the female bovine. Both androgens gave the purple Zimmermann color characteristic of 17-ketosteroids. The digitonin-precipitable androgen is tentatively regarded as an androstane (ene) derivative containing a 3β-hydroxyl group and a 17-keto group. Similarly, the digitonin-nonprecipitable androgen is tentatively regarded as an androstane (ene) derivative containing a 3α-hydroxyl group and a 17-keto group.

The experimental evidence established that the biliary androgens in the female bovine are in a conjugated state, whereas the fecal androgens are in an unconjugated state.

The fact that the biliary androgens are hydroxyketones, as compared to the diketonic fecal androgens, suggests an enterohepatic recycling of the androgens in the bovine or alteration by the microorganisms of the gastro-intestinal tract.

Some years ago, it was discovered that sexually mature dairy cows excrete large quantities of androgens in the feces (8, 21, 25, 26), whereas sexually mature bulls do not. Maximum elaboration of the fecal androgens during the normal estrous cycle of the cow showed a peak coincident with corpus luteum maturation. Moreover, feces from pregnant cows contained the greatest amount of androgenic activity.

These and other observations led to the conjecture that the androgens in bovine feces might be metabolites of progesterone. Consequently, progesterone was injected into the bovine and the feces collected before, during, and after progesterone administration. The results demonstrated that the androgens found in the feces of the sexually active female bovine are metabolites of progesterone and can be significantly increased upon injection of progesterone (17). The in vivo transformation of progesterone to androgens was demonstrated in the bull, also. Following the administration of progesterone to a pregnant cow, the

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androgens androstane-3,17-dione, $\Delta^{1,4}$-androstadiene-3,17-dione, and Δ^{4}-androstene-3,17-dione were isolated from the feces, together with etiocholane-3,17-dione (18) (Figure 1).

Many recent in vitro reports have indicated the important role of progesterone in androgen biosynthesis via the following pathway: progesterone → 17 α-hydroxyprogesterone → Δ^{4}-androstene-3,17-dione (androstenedione).

The biogenesis of androstenedione from progesterone via 17 α-hydroxyprogesterone has been demonstrated in various mammalian tissues, including adrenal, testis, and ovary, and peripheral tissue, of which the liver is unquestionably the most important. Solomon *et al.* (24) demonstrated the conversion of progesterone to 17 α-hydroxyprogesterone and androstenedione by bovine ovarian homogenates. Lynn (13) demonstrated that particulates from bovine testicular tissue

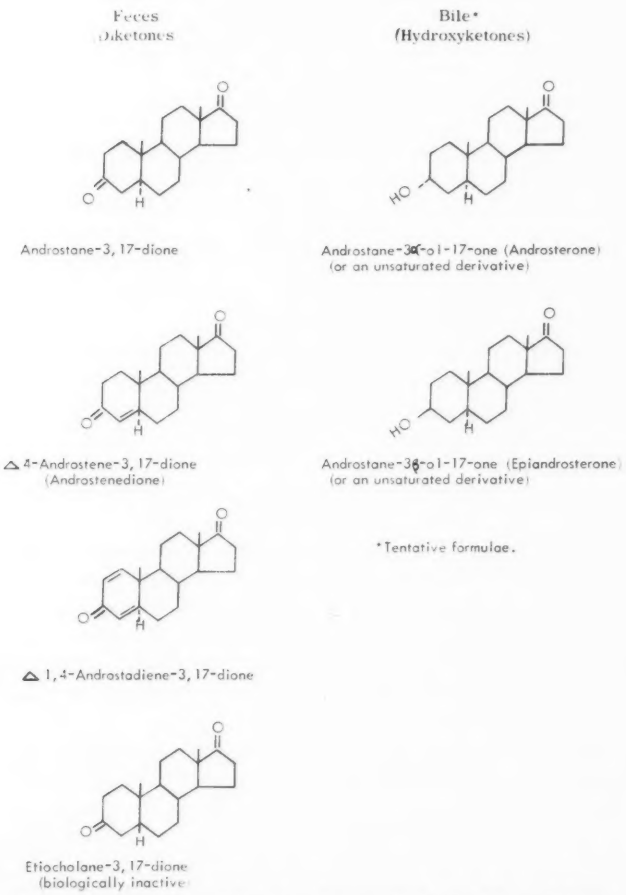

FIG. 1. Structural formulae of androgens in bovine bile and feces.

could convert progesterone or 17 α-hydroxyprogesterone to androstenedione.

Various experiments with labeled progesterone have demonstrated the major role of the liver and bile in the metabolism and excretion of the metabolites of progesterone in the rodent (1, 10, 11, 20, 23) and human (2, 4, 5, 7, 22, 27). In addition, large amounts of hitherto unrecognized metabolites of progesterone were indicated in the bile and feces. Pearlman (19) indicated the importance of the biliary pathway for the elimination of progesterone metabolites in pregnant cows. The presence of androgens has been demonstrated also in bovine bile (14, 17).

In an attempt to further elucidate the hepato-biliary-enteric metabolism, recycling, and excretion of steroids in the bovine, the present study describes the hydrolysis, extraction, and fractionation of bovine bile and the subsequent identification of the properties and functional groups of the biliary androgens.

EXPERIMENTAL PROCEDURE

Collection, hydrolysis, and extraction of bile. Three 20-liter lots of bovine bile from sexually mature female dairy cattle were obtained from the slaughterhouse. A preliminary study indicated that the biliary androgens were very largely in a conjugated state, since they were not extractable with ether before the bile was hydrolyzed (17). Consequently, the bile was hydrolyzed by adding 15% by volume of concentrated hydrochloric acid and the mixture refluxed for 15 min. After cooling to room temperature, the hydrolysates were extracted several times with ethyl ether. The ether extracts were washed with distilled water until neutral, dried over sodium sulfate, and the ether concentrated by distillation to a volume of approximately three liters.

Fractionation of extracts. Following ether extraction of the bile hydrolysates, the ether-soluble material was fractionated and the androgens traced by bioassay as follows: (a) separation into the acidic and neutral fractions; (b) separation of the neutral fraction into the alcoholic and nonalcoholic fractions; (c) separation of the neutral alcoholic fraction into the ketonic and nonketonic fractions; (d) separation of the neutral alcoholic ketonic fraction into the alpha and beta fractions.

Biological assay method. The pathways of the androgens were traced in the fractionation procedures by bioassaying aliquots of the various fractions resulting from the separation procedures. To conserve material, not every fraction obtained in the processing of each 20-liter lot of the bile was bioassayed. However, a sufficient number of fractions from the three lots of bile were bioassayed qualitatively, to establish the pathway of the androgens in the separation procedures.

The bioassay method used was the modified chick comb application method previously described (16). Groups of seven-day-old White Plymouth Rock cockerels were used as assay animals. The material to be assayed was dissolved in sufficient solvent so that each chick in the group received a daily comb application of 0.05 ml. for ten days. Twenty-four hours after the last application the chicks were autopsied. Comb response was expressed as 100 times the ratio of comb weight in milligrams to body weight in grams.

As a measure of the presence of the biologically active androgens in the various fractions, comparison was made between the average comb ratios of groups of treated chicks and the average comb ratio of a group of control chicks.

Separation of the neutral and acidic fractions. The procedure for the separation of the ether-soluble material into neutral and acidic fractions was that used by Dobriner *et al* (6). Each ethereal solution was extracted several times with 2 *N* sodium hydroxide solution. The alkaline washings were back-extracted with ether. The combined neutral ethereal solutions were washed with water and dried over sodium sulfate. The neutral fraction was obtained by evaporation of the ether under reduced pressure.

The alkaline washings were acidified and then extracted several times with ether. The ether was dried and distilled under vacuum. The residue was the acidic fraction.

Bioassay of each residue revealed the presence of the androgenic factor in the neutral fraction (Table 1).

TABLE 1

Biological estimation of the biliary androgens in the neutral and acidic fractions

Lot No.	Fraction	No. of cockerels	Av. body wt. *(g.)*	Av. comb wt. *(mg.)*	Comb ratio
	Control	9	191.7	70.4	36.7
1	Neutral	10	182.4	197.9	108.5
1	Acidic	10	191.0	87.8	35.5

The neutral fraction was further purified by partitioning between 70% ethanol and heptane. The alcohol portion was extracted twice with equal volumes of heptane, and the combined heptane extracts were then backwashed with one volume of 70% ethanol. The combined ethanol extracts and the combined heptane extracts were separately distilled to dryness.

By distribution of the neutral fraction between 70% ethanol and heptane, most of the lipid material was removed in the heptane phase. Bioassay of an aliquot of the heptane-soluble material demonstrated the absence of androgens in this phase. The ethanol-soluble material from the neutral fraction was further purified.

Separation of alcoholic and nonalcoholic neutral fractions. The neutral fractions were dissolved in pyridine and refluxed for 3 hr. with twice their weight of succinic anhydride (28). The alcoholic hemisuccinates were removed from the nonalcohols by extracting an ethereal solution of the constituents with sodium carbonate solution. The alcohols were removed from their esters by refluxing for 1 hr. in 3.3 *N* aqueous ethanolic potassium hydroxide solution. The androgen content of the alcoholic and nonalcoholic fractions was determined routinely. The alcoholic fraction was active biologically, whereas the nonalcoholic fraction was inactive (Table 2). This denoted the presence of a functional hydroxyl group within the structure of the androgen molecule.

TABLE 2

Biological estimation of the biliary androgens in the alcoholic and nonalcoholic neutral fractions

Lot No.	Fraction	No. of cockerels	Av. body wt. *(g.)*	Av. comb wt. *(mg.)*	Comb ratio
	Control	10	154.3	63.4	41.1
1	Alcoholic	9	134.3	88.3	65.8
1	Nonalcoholic	9	146.7	59.6	40.7
	Control	11	155.4	53.1	34.2
2	Alcoholic	14	162.6	87.5	53.8
2	Nonalcoholic	15	165.8	45.6	27.5
	Control	10	149.6	49.1	32.8
3	Alcoholic	9	148.8	112.7	75.8
3	Nonalcoholic	9	145.3	58.8	40.5

Separation of ketonic from nonketonic neutral alcohols. The neutral alcoholic fractions were separated into ketonic and nonketonic fractions by means of Girard's Reagent T (9) in the usual manner, using 15 ml. of absolute ethanol, 1.5 g. of glacial acetic acid, and 1.5 g. of the reagent for every gram of the neutral residue. The ketones were recovered from the hydrazones by acid hydrolysis at room temperature for 24 hr., followed by extraction with ether.

The fractions were bioassayed as before. The ketonic fraction exhibited androgenic activity (Table 3). This indicated the presence of a functional carbonyl group within the androgen molecule.

TABLE 3

Biological estimation of the biliary androgens in the ketonic and nonketonic neutral alcoholic fractions

Lot No.	Fraction	No. of cockerels	Av. body wt. *(g.)*	Av. comb wt. *(mg.)*	Comb ratio
	Control	10	157.0	34.7	22.3
3	Ketonic	10	152.0	105.0	70.4
	Control	12	172.6	46.5	27.0
3	Nonketonic	12	165.8	54.5	33.3

The neutral hydroxyketonic fractions obtained from processing the three lots of bovine bile were combined for further purification.

Digitonin separation of neutral alcoholic ketones. The method for the digitonin separation of the neutral alcoholic ketones into alpha and beta fractions was based on the modification by Dobriner *et al.* (6) of the procedure of Butler and Marrian (3). The digitonides were precipitated from a 90% ethanol solution in which the concentration of ketosteroid was 1% and that of the digitonin was 1.2%. The 3β-epimers were liberated from their complexes by heating with pyridine.

The digitonin-nonprecipitable (α) fraction weighed 460 mg. and contained an amount of 17-ketosteroids equivalent to 35 mg. of androsterone, as determined by the modified Zimmermann reaction (12). The digitonin-precipitable

(β) fraction weighed 12 mg. and contained "2.5 mg. equivalents of dehydroisoandrosterone" as measured by the Zimmermann reaction.

Aliquots of these fractions were bioassayed for androgenic activity (Table 4). Since both fractions were active, the results indicate the presence of a digitonin-precipitable and a digitonin-nonprecipitable androgen in bovine bile.

TABLE 4

Biological estimation of the biliary androgens in the fractions resulting from digitonin separation of the hydroxyketones

Lot No.	Fraction	No. of cockerels	Av. body wt. (*g.*)	Av. comb wt. (*mg.*)	Comb ratio
	Control	10	119.0	33.7	20.0
1–3	α-Hydroxyketones	10	116.6	86.8	74.4
	Control	12	172.6	46.5	27.0
1–3	β-Hydroxyketones	12	169.0	92.7	54.9

RESULTS

The fractionation procedure identified a digitonin-precipitable and a digitonin-nonprecipitable hydroxyketonic androgen from the neutral, saponifiable material of bile from the bovine female. The androgens gave a purple Zimmermann color characteristic of 17-ketosteroids. The digitonin-precipitable androgen is tentatively regarded as an androstane (ene) derivative containing a 3 β-hydroxyl group and a 17-keto group. Similarly, the digitonin-nonprecipitable androgen is tentatively regarded as an androstane (ene) derivative containing a 3 α-hydroxyl group and a 17-keto group (Figure 1).

DISCUSSION

It is tempting to speculate as to the complete identity of these biliary androgens. The most likely presumptive conclusions seem to be epiandrosterone (or dehydroepiandrosterone) and androsterone.

The experimental evidence established that the biliary androgens in the female bovine are in a conjugated state, since they were not extractable with ether before hydrolysis of the bile. In contrast, it has been established that fecal androgens are unconjugated (15). The biliary androgens have a hydroxyl group available for esterification as the sulfate or glucuronidate, whereas the absence of the hydroxyl group in the diketonic fecal androgens eliminates the possibility of their conjugation by either of these modes of steroid conjugation. These results are in accord with recent reports demonstrating that the biliary metabolites of administered labeled neutral steroids in the rodent (1, 10, 11, 20, 23) and human (2, 4, 5, 7, 22, 27) are in a conjugated state, whereas the fecal metabolites appear almost exclusively in the nonsaponifiable material of feces.

Since the androgens previously identified in the feces of a pregnant cow are metabolites of progesterone (17, 18), the androgens in the bile of the female bovine are presumed to be metabolites of progesterone. It is interesting to note that the biliary androgens are hydroxyketones, as compared to the diketonic

fecal androgens. Since the hydroxyketones are regarded as catabolites of the diketones in steroid metabolism, these observations suggest an enterohepatic recycling of the androgens or an alteration by the microorganisms of the gastrointestinal tract.

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TECHNICAL NOTES

EFFECTS OF FREQUENCY OF FEEDING UPON RUMEN VOLATILE ACIDS, PROTOZOAL POPULATION, AND WEIGHT GAINS IN ANGUS HEIFER CALVES

Feeding more frequently than twice daily has improved weight gains in sheep (1, 11) and in cattle (6, 7, 10). There have also been reports where no response in weight gains were observed (3, 5). Increased feed consumption may explain the improved gains in some instances (7), but not when the animals are pair-fed (1, 6, 10). The proportions of volatile fatty acids were not affected by frequent feeding according to Leffel and Komarek (5), but Mochrie (6) reported less diurnal variation in volatile fatty acid concentrations when animals were frequently fed. Knox and Ward (4) observed higher volatile fatty acid concentrations and a lower acetic/propionic ratio when animals were fed frequently. Moir and Somers (8) observed increases in the rumen population of oligotrich protozoa when sheep were fed four times versus one time daily. Rakes *et al.* (11) have observed less heat production by sheep which were frequently fed. The present work was conducted to investigate further the effects of frequency of feeding upon weight gains, rumen volatile fatty acid concentrations, and the rumen protozoal population.

Eight Aberdeen Angus heifer calves ranging in weight from 253 to 368 lb. were used as experimental animals. They were paired according to body weight and randomly allotted to the twice-a-day and ten-times-a-day feeding schedules. The animals were individually penned and fed U.S. No. 2 alfalfa hay which had been ground through a 1½-in. screen. Salt, bone meal, and water were available at all times. A 4-wk. adjustment period preceded the two, 87-day experimental periods. The treatment groups were reversed during the second period. The feed intakes were adjusted by pairs as the experiment progressed. The feed offered each pair was limited to the amount that would be consumed without weighbacks, regardless of differences in apparent live weight. The animals in one group were fed equal portions at 8 A.M. and 5 P.M. (2 ×), whereas the animals in the other group were fed equal portions each hour from 8 A.M. through 5 P.M. (10 ×). Fasted (18 hr.) weights were taken at the beginning and end of each period and full weights were taken weekly. The animals were weighed at 12:30 P.M. Rumen samples for volatile fatty acid analyses were taken twice during each experimental period by means of a stomach tube. Samples were obtained from 8:30 A.M.—9 A.M. The volatile fatty acids were determined by means of a silicic acid type chromatographic column (9). Differential counts of the rumen protozoal population (2) were made at 2-wk. intervals during the first 8 wk. of the experiment.

As illustrated in Table 1, the frequently fed animals made significantly greater gains than the animals fed twice daily. The fasted weights

TABLE 1

Effect of frequency of feeding on body weight gain, volatile fatty acid concentration in rumen liquor, and protozoal counts

	Frequency of feeding		Difference due to frequent feeding	Standard error of difference
	2 ×	10 ×		
Feed consumed per unit of live weight gain *(lb.)*	17.6	12.3	−5.3	2.95
Body weight gain per animal *(lb/period)*	48.1	64.8	16.7[a]	5.05
Volatile fatty acid concentration				
Acetic, m*M*/liter	42.1	46.5	4.4	7.72
molar %	67.7	67.1	−0.6	1.61
Propionic, m*M*/liter	11.0	12.6	1.6	1.66
molar %	17.5	17.2	−0.3	0.62
Butyric plus higher acids, m*M*/liter	9.5	11.0	1.5	1.72
molar %	14.8	15.7	0.9	1.15
Total acids, m*M*/liter	62.6	70.1	7.5	8.86
Protozoal counts				
log *Entodinium*/milliliter	2.048	2.602	0.554[b]	0.165

[a] and [b] Indicate statistical significance, $P < 0.05$ and $P < 0.01$, respectively.

were used for these calculations. The over-all advantage was similar when the full weights were used, but was less consistent between pairs. Due to the paired feeding, feed intakes were the same; however, the frequently fed animals appeared to have greater appetites. The 2 × animals usually consumed their feed within an hour, whereas the frequently fed animals consumed their smaller portions immediately. Both groups consumed an average of 2.4 % of their body weight daily.

The average volatile fatty acid concentrations appear somewhat greater for the 10 × animals (Table 1). However, within-pair comparisons were inconsistent and the difference does not approach statistical significance. The values observed during the preliminary period were also similar.

The average protozoal counts for the frequently fed animals were 12.3×10^5 entodinia per milliliter of rumen contents at the beginning of the experimental period and 26.2×10^5 per milliliter after 8 wk. The average for the 2 × group was 15.6×10^5 entodinia per milliliter at the beginning and 14.1×10^5 per milliliter at the end of the same period. The difference in populations (Table 1) was highly significant ($P = 0.01$). The small oligotrich protozoan, *Entodinium*, was the only ciliate which showed an increase in population with animals on the frequent feeding regime. The protozoan genera, *Isotricha*, *Ophryoscolex*, *Dasytricha*, and *Diplodinium*, were found in low numbers or were entirely absent in both the experimental and control groups.

The observed increase in body weight gains when animals were frequently fed, supports previously reported work with sheep and cattle. The increase in the population of the protozoan, *Entodinium*, accompanying frequent feeding is in accord with the observations of Moir and Somers (8) with sheep, and suggests that changes in the microbial population are related to changes in feeding management, even though the animals may be consuming equal amounts of the same ration daily. On the contrary, there was no apparent relationship between frequency of feeding and volatile fatty acid concentrations or ratios as measured.

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BLOAT IN CATTLE. III. COMPOSITION OF FOAM IN LEGUME BLOAT[1]

Most investigators consider excessive foaming of rumen ingesta following consumption of legumes as the primary factor in the etiology of bloat. Cole and Boda (2) indicated divided opinion on what chemical substances cause foaming. Plant proteins, saponins, and pectic substances appear to be involved. Also, the slime produced by bacteria has been suggested as a factor in frothing of rumen ingesta. Head (4) observed that constituents responsible for foaming in cows eating clover and alfalfa were carbohydrate, the main constituents being the sugars normally found in the pectin and hemicellulose fractions of plants. Head stated: "This observation suggested that the foaming constituent appeared in solution in the rumen liquor after the plant had been physically broken down, and that it later disappeared, probably by the degrading action of the microorganisms."

[1] Contribution No. 294, Department of Dairy Husbandry, Kansas Agricultural Experiment Station, Manhattan, Kansas.

Since results of studies at this station (1) suggested that microflora are involved in frothy bloat, and that bloat is not the result of simple physical breakdown of feed, further analyses of the froth might refine the relative importance of each of these factors.

Nine cows fitted with rumen fistulas were pastured on bloat-provoking alfalfa. During periods of maximum bloat, rumen foam was collected in 5-gal. containers and strained through two layers of cheese cloth. The procedure used to isolate the foaming constituents was essentially that of Head (4). The strained fluid was treated with Supercel (30 ml. rumen fluid to 1 g. Supercel), stirred, and then filtered through Whatman No. 4 filter paper, using a Büchner funnel and water aspirator. The filtrate foamed on shaking. The filtrate was adjusted to pH 4 with dilute HCl and the foaming constituent was precipitated by adding absolute alcohol (70 ml. alcohol to 30 ml. filtrate). After centrifugation at 800 × G for 10 min., the precipitate was removed and dissolved in distilled water. The solution of the precipitate revealed the following composition percentages: protein (N × 6.25) 63.3, ether extract 1.5, crude fiber 0, carbohydrates 17.0, ash 18.2.

Paper chromatography was used to determine the amino acid composition of the precipitate. The precipitate was hydrolyzed with 2 *N* HCl for 6 hr. The solution was neutralized with NaOH, filtered, and then passed through a Dow X-50 resin column to remove salts. Amino acids eluted from the column were then analyzed, using the method of Consden *et al.* (3). The following amino acids were identified: aspartic acid, alanine, arginine, glutamic acid, glycine, histidine, hydroxyproline, leucine, lysine, methionine sulfone, serine, tyrosine, valine, phenylalanine, and threonine.

The conclusion that the foaming constituent is primarily proteinaceous is not in harmony with Head's results (4). He observed that the constituent responsible for foaming in rumina of cows eating clover and alfalfa was carbohydrate. Since the method employed for isolating the foaming constituents was essentially Head's, it is apparent that the difference in the two results must be attributed to differences in the two foams analyzed. Because the nature of the foaming agent may throw some light on the cause of bloat, further work on changes in foam composition during bloat is indicated.

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NEW TECHNIQUES FOR RECORDING DHIA DATA UNDER FIELD CONDITIONS [1, 2]

Electronic computers and related peripheral punch-card equipment are in common use in the machine processing of DHIA records. Approximately 38% (150,000) of the DHIA cows in Wisconsin are presently enrolled in machine processing. This system requires the key-punching and verification of data reported from the field each month and is a costly operation. At the Wisconsin Agricultural Records Cooperative, from 20 to 24% of the total machine processing cost, or approximately 2.0¢/cow/month, is attributed to key-punching and verification. This study was made in an attempt to determine the feasibility of using other methods of assembling data for machine processing.

Recording data by mark sensing has been widely used by many industries. This system requires the use of a mark-sense card, an electrostatic pencil, and a mark-sense device in an IBM reproducer. More recently, the method of port-a-punching was introduced and used successfully in certain industries. This system requires a port-a-punch card, board, and punch, and differs from mark sensing in that punches rather than marks are originally recorded. Each of these systems was tried in this study, along with the barn-sheet method of machine processing.

A preliminary trial involving eight herds during 4 mo. was made to develop card formats and

[1] Contributions from the Dairy Cattle Research Branch, USDA, Beltsville, Md., and from Wisconsin's Agricultural Records Cooperative.

[2] Approved for publication by the Director of the Experiment Station, University of Wisconsin, Madison.

to gain experience in recording and processing. The formats of the two cards included daily milk weight, per cent fat, pounds of grain, and change of status codes and dates. To facilitate subsequent handling by field personnel, herd code, cow name and number, last change of status code, and date and production last month were pre-punched and printed on the cards. The time required to key-punch and verify an average 32-cow herd was 6.0 min., whereas mark sensing and port-a-punching required 16.9 and 13.4 min., respectively. The key-punching was done by experienced personnel.

In the field trial, a double-recording system was used during 7 mo. in five standard DHIA herds which were serviced by five supervisors. In addition to the normal barn-sheet method, mark-sense cards were used during the first 4 mo. and the port-a-punch during the last 3 mo. Recording was done in the barn at the time of milking. On the 1st and 5th mo., a research assistant used the special cards, and the supervisors recorded on the barn sheet. During other months, the procedure was reversed. In the DHIA laboratory the fat column of each sample was read and recorded on the barn sheet. When the samples from each herd were completed, the procedure was repeated and the tests recorded on the special cards. This was done in order to more accurately appraise the time required to utilize the two systems.

The mark-sense and port-a-punch cards were compared with the daily production card, which was key-punched from the barn sheet. Eleven of 620 mark-sense cards (1.9%) and 23 of 301 port-a-punch cards (7.6%) did not agree with the cards resulting from normal machine processing. It was assumed that the cards key-punched from the barn sheets were correct. The apparent mark-sense errors were distributed over all variables recorded, whereas 16 of the 23 port-a-punch errors involved milk weights, most of which occurred during the 1st mo. One of the five supervisors was responsible for three and 11 of the discrepancies in the mark-sense and port-a-punch cards, respectively. In addition to these, missing information accounted for 8.6 and 3.0% of the total mark-sense and port-a-punch cards, respectively. This resulted largely from failure on the part of field personnel to record zero production for dry cows and change of status codes and dates.

It was generally evident that under barn conditions the mark-sense cards were easier to score than the port-a-punch cards. The use of the two card systems at both the barn and testing laboratory was more time-consuming than the use of the barn sheet. In the laboratory, technicians read the fat column and recorded percentage of fat on the barn sheet in 4.4 min. per herd. The corresponding time required for the mark-sense and port-a-punch methods was 6.7 and 8.6 min., respectively. While it is assumed that the efficiency in the use of the card methods would increase with experience, it is doubtful if this would compensate for the time required to sort the cards in order by sample number. Time trials at the farm were more difficult to appraise. It was clearly evident, however, that the necessary sorting of cards almost doubled the recording time, as compared with the barn-sheet method. The mark-sense card was easier to handle than the port-a-punch card.

No great difficulty was experienced in machine processing the cards from the two systems. However, machine failure in detecting mark senses and port-a-punches involved 0.5 and 1.0% of the cards, respectively.

It was not possible to determine with accuracy the cost of the two card systems. Assuming a monthly volume of 150,000 cows, it was estimated that a shift from conventional key-punching and verifying to either of the other methods would increase the cost per cow per month by .25¢ in the machine room, .33¢ in the handling of incoming and outgoing materials, and from .50¢ to 1.00¢ for mailing, while reducing key-punching costs by approximately 2¢. Thus, the net savings would be less than 1¢/cow/month. Since this trial did not provide for the processing of new cows and herds, and for other data necessary for normal DHIA record-keeping, this estimated cost advantage would be further reduced.

The only apparent advantage of either card system over the method of key-punching and verifying of barn sheets was in the reduction of machine-processing costs. It is likely that this saving would not exceed .5 to 1¢/cow/month. It is doubtful if the quality of the DHIA supervisor or the degree of his supervision is adequate for either of the card systems. Their use in owner-sampler herds did not seem advisable. Of the two systems, mark sensing appeared superior to port-a-punching. It could be effectively used in the collection and recording of data by qualified or closely supervised personnel.

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ASSOCIATION BETWEEN CEREBROSPINAL FLUID PRESSURE AND PLASMA VITAMIN A CONCENTRATION OF HOLSTEIN CALVES FED FIXED INTAKES OF CAROTENE [1]

Cerebrospinal fluid (CSF) pressure of normal cattle to 2 yr. of age, measured at the atlanto-occipital articulation, averaged 105 mm. saline (0.85%), with a range of values between 80 and 150 mm., and was unaffected by age, Sykes and Moore (9). When graded levels of carotene intake were fed to calves, increases in pressure above 120 mm. occurred at intakes below 30 γ of carotene per pound of live weight per day for Ayrshires and Holsteins, and below 34 and 32 γ, respectively, for Guernseys and Jerseys (6, 7). The increased CSF pressure appeared to be the first change occurring in experimentally produced vitamin A deficiency in the calf and several other species, as reviewed recently (2). Also, plasma vitamin A concentrations, under conditions of fixed carotene intake, were proportional to the logarithm of carotene intake (10), thus indicating a possible association between CSF pressures and plasma vitamin A concentrations.

Graphic representation of CSF pressure against plasma vitamin A concentrations, for data collected at the Storrs Station from 1950 to 1957, described a region of decreasing CSF pressures in the range of low to medium plasma vitamin A concentrations (approximately 4 to 16 γ per 100 ml.). However, as plasma vitamin A concentrations increased above the medium values, a region of constant pressure was entered, due to the maintenance of normal CSF pressure. Since these measurements of CSF pressure could not discriminate among values of less than the height from the point to the hub of the puncture needle (90 mm.), because of the use of a rigid glass or polyethylene tube (7), the quantitative aspects of the above association between the two variables could not be adequately studied. More recently, CSF pressures have been measured manometrically, providing measurements over the range of possible pressures. The present study was undertaken to ascertain the regression of CSF pressure on plasma vitamin A concentration.

The statistics reported herein were derived from the data of 91 Holstein male calves employed in experiments conducted at the Storrs Station during the period 1958-1960. These calves, previously raised on limited whole milk or milk replacer, limited calf starter or grain, and ad libitum chopped alfalfa hay to approximately the 63rd day of age, were fed a vitamin A depletion ration at levels of intake to provide an anticipated seven-day increase in live weight of 10 lb. After the plasma vitamin A concentration had decreased to 12.0 γ or less per 100 ml., each calf was fed a fixed intake of carotene from artificially dehydrated alfalfa (86 calves) or from water-dispersible gelatin beadlets (five calves). The range of carotene intakes was between 10 and 240 γ per pound of live weight per day and the period of carotene supplementation was either 12, 16, or 24 wk. The plasma vitamin A concentrations determined by the Kimble procedure (4) and the CSF pressures determined manometrically (2, 9) represent values obtained three to ten days prior to completion of each calf's designated carotene supplement period. In the subsequent analysis of these data, no attempt was made to isolate variation due to experiments or to duration of carotene supplementation. However, durations of 12 or 24 wk. apparently

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TABLE 1

Association between plasma vitamin A concentration and cerebrospinal fluid pressure in Holstein calves fed fixed intakes of carotene

Plasma vitamin A concentration			Cerebrospinal fluid pressure			
			Actual		Common logarithm	
Interval	Actual mean	Calves	Mean	Standard deviation	Mean	Standard deviation
——(γ/100 ml.)——		(No.)	——————(mm. of saline)——————			
0.0– 3.9	3.4	1	355	....	2.55	....
4.0– 7.9	6.1	16	200	91	2.26	0.19
8.0–11.9	9.8	16	130	69	2.06	0.22
12.0–15.9	13.8	24	93	34	1.94	0.16
16.0–19.9	17.6	12	76	23	1.87	0.12
20.0–23.9	21.6	13	74	23	1.86	0.12
24.0–27.9	25.0	7	65	14	1.81	0.10
28.0–31.9	31.3	1	80	....	1.90	.....
32.0–35.9	32.0	1	58	....	1.76	.....

have inappreciable effect on CSF pressure under conditions of fixed intakes from 10 to 60 γ of carotene (3).

Table 1 presents the mean CSF pressures at intervals of 4.0 γ per 100 ml. of plasma vitamin A concentration. Rather marked decreases occurred in the CSF pressure as plasma vitamin A concentration increased to and including the interval 12.0 to 15.9 γ per 100 ml. Thereafter, there was little change. The standard deviations of the CSF pressure measurements increased as the average magnitude of this measurement increased (Table 1, Column 5). Since the standard deviations appeared to be approximately proportional to the means, the CSF measurements were transformed (8) to their respective common logarithms (Table 1, Columns 6 and 7). The transformation reduced the differences in variation of CSF pressure among plasma vitamin A concentration intervals, since Bartlett's homogeneity of variance test (8) was $P < 0.001$ for the variances of actual CSF values and only $P < 0.10$ for those of the transformed values.

To quantify the association between CSF pressure and plasma vitamin A concentration, a broken-line regression equation was fitted to the data by the method of least squares (Figure 1). For plasma vitamin A concentrations above 15.6 γ per 100 ml., CSF pressure was maintained at an average level of 1.85 logarithmic units, cor-

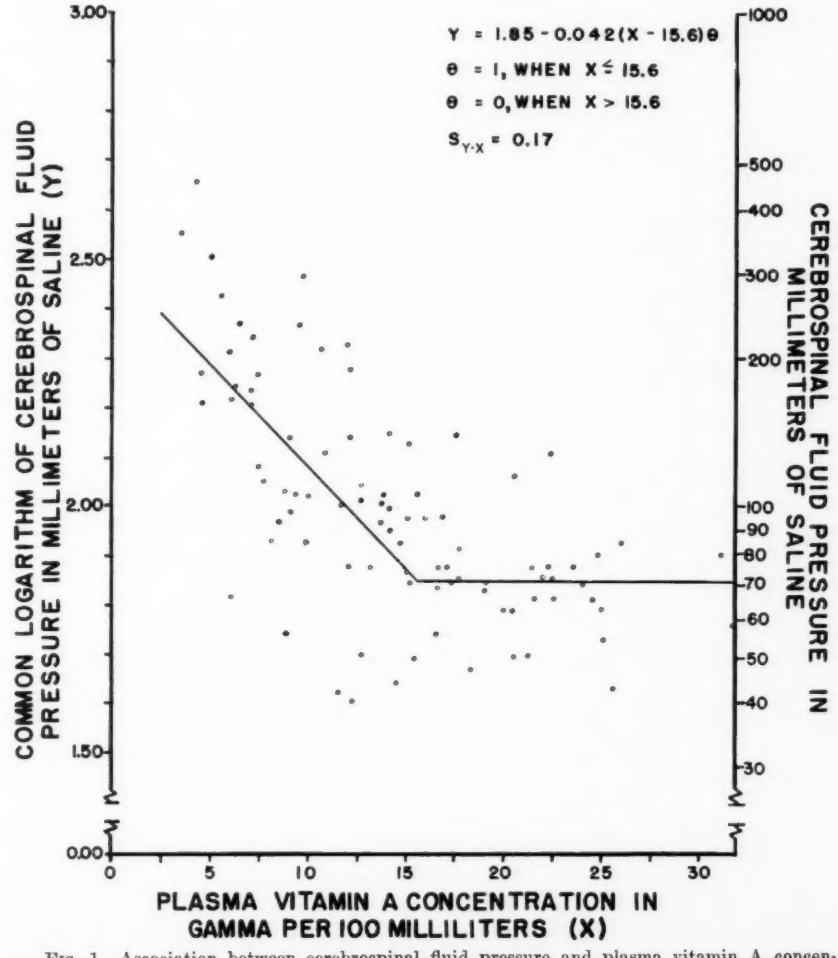

FIG. 1. Association between cerebrospinal fluid pressure and plasma vitamin A concentration of Holstein calves fed fixed intakes of carotene, as represented by a broken-line regression equation.

responding to a pressure of 71 mm. saline. The possibility of a nonzero slope in the region of plasma vitamin A values greater than 15.6 γ concentration was investigated, but did not account for a significant portion of the variation. As concentration of plasma vitamin A increased from the lowest observed value, 3.4 γ per 100 ml., to 15.6 γ, CSF pressure was found to decrease 0.042 log unit per gamma increase in plasma vitamin A concentration. The sloping portion of the broken line intersected the ordinate at 2.50 log units.

The plasma vitamin A concentration of 15.6 γ per 100 ml., which separated the region of change from that of little or no change of CSF pressure, agreed reasonably well with a value of 18 γ of vitamin A per 100 ml. of plasma, based on reproductive performance of beef cattle fed carotene intakes between 66 and 528 γ per pound of live weight per day (5), rather than on CSF pressure. In the latter study (5), a spectrophotometric method for estimation of plasma vitamin A was used; in the present study, the Carr-Price reaction (4) was used, which has been shown to give lower vitamin A values (1). The value of 71 mm. obtained for the CSF pressure in the region of no change cannot be compared directly with the average of 105 mm. for normal cattle given by Sykes and Moore (9), because the former is analogous to a geometric mean, which gives relatively less weight to high values. The arithmetic mean for the pressures in the region of no change in the present study was 74 mm., certainly lower than the 105 mm. average, but the reasons for this difference are not presently clear. Also, while biological considerations give strong support to the broken-line regression as being representative of the approximate functional relationship between CSF pressure and plasma vitamin A concentration, the data of this study are inadequate to discriminate between the broken-line regression and many other conceivable relations.

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EFFECTS OF THE MALE HORMONE ON THE FEMALE GENOTYPE

To further clarify the role of the sex hormones in growth and development of the bovine, quantitative effects of the male hormone on the female genotype were studied. Although the effects of castration and consequent removal of most of the male hormone supply on the young male are qualitatively well-known (1), the effects of the male hormone on the female are but little understood.

One of the major difficulties in quantitative biological studies is the control of genetic variation. By using identical twins which have, by definition, identical genotypes, this source of variation is minimized (2).

The identical twin Brown Swiss freemartins (triplet to a bull) used in this study exhibited the palpable testes, small vagina, enlarged clitoris, and coarse vulvar hair characteristic of freemartins. At 4 mo. of age, a laparotomy was performed [1] on both, which revealed an underdeveloped uterus, no cervix, dangling fallopian tubes, and no ovaries, but histologically normal testes. That the twin freemartins were monozygotic and, therefore, genetically identical seems certain from lack of dissimilarities of nose prints, hair color, hair patterns, temperaments, blood antigen patterns based on more than 40 antigens, and closely similar body dimensions during their first 12 mo. The only differences noted were that one had a white tongue, while the other had a black tongue, and some differences in anatomical dimensions, probably as a result of unilateral gonadectomy of the experimental twin at 4 mo. of age.

After uniformity studies were conducted, the other testicle was removed from the experimental freemartin at 13.5 mo. and the effects noted.

At 19.5 mo. of age, testosterone propionate [2] in sesame oil was administered intramuscularly into the neck of one freemartin. For 16 wk., the average dose was about 18 mg/day. Then for 2 wk., 100 mg/day was given. Two weeks later, at 24.5 mo. of age, the animals were slaughtered.

Nineteen measurements were made weekly (3). Some pertinent data are presented in Table 1. Androgens caused increases in the body weight and circumference of neck and increases in heart girth, depth of chest, width of shoulders, distance from stifle to stifle (posteriorly measured), width at thurls, length and width of head, and circumference of muzzle. The height at withers, length of body, circumference of metatarsi and metacarpi, width at hooks, width at pins, hooks to pins, and mandible width were relatively unaffected in this animal. No statistical test is available for testing differences between these data. The trends, however, with the reversals of certain measurements intended to assess various tissues coinciding with treatments strongly support the conclusions drawn. It was concluded that androgens at moderate levels affect the female genotype in causing increased muscle and growth in flat bones. In over-all measurements also, the effect is similar to that of the male.

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[1] Through assistance offered by Dr. V. L. Tharpe and Dr. C. R. Cole, College of Veterinary Medicine.

[2] Supplied by Ciba Pharmaceutical Products, Inc.

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TABLE 1

Body measurements of the identical Brown Swiss freemartins

Experimental period		Control	Deprivation effects		Restitution effects	
Age [a]		12 mo.	15 mo.	18 mo.	21 mo.	24 mo.
Measurement						
Body weight	E	708	786	859	929	1027
(lb.)	C	706	754	836	939	1052
Circumference neck	E	90.7	87.7	94.2	95.3	94.9
(cm.)	C	90.3	85.6	91.2	95.0	97.0
Heart girth	E	154.4	159.5	166.8	170.6	178.2
(cm.)	C	154.4	157.6	164.9	170.1	179.1
Chest depth	E	53.9	57.2	60.3	62.0	64.7
(cm.)	C	53.5	56.3	59.6	61.4	64.5
Shoulder width	E	37.3	38.4	40.3	41.5	43.9
(cm.)	C	38.1	38.0	40.5	42.1	44.8
Withers height	E	117.6	122.6	127.4	129.7	131.8
(cm.)	C	117.9	123.0	127.4	129.3	132.1
Body length	E	121.3	122.6	127.4	129.7	131.8
(cm.)	C	121.1	123.0	127.4	129.3	132.1

[a] The ages and comparison are the means for 13 wk., including 6 wk. preceding and 6 wk. following the week representing the age given.

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600	37.50	51.25	93.75	137.50	177.50	221.25	266.25	313.75	346.25
700	41.25	56.25	105.00	153.75	197.50	246.25	297.50	350.00	387.50
800	45.00	62.50	115.00	168.75	218.75	272.50	328.75	386.25	427.50
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BOOK REVIEW

Leche, su Producción Higienica y Control Sanitario (Milk, Its Hygienic Production and Sanitary Control) (In Spanish). By Dr. Mario Ramos Cordova, Head of Laboratory of the National Association of Pure Milk Producers, Mexico City, Mexico. VI. 251 pp. Numerous tables, figures, and references. Mexico City. Published by the author, Reforma No. 330 (1960). Price U. S., $8.

The book is divided into two sections. The first one deals with the sanitary production of milk, and factors affecting its chemical composition. The different sources of contamination are discussed from the interior of the udder and from external sources involving saprophytic and pathogenic bacteria and their control, including the essentials of sanitary milking practices. The cleaning and sterilizing of utensils, pails, cans, and dairy processing equipment; milk-filtering, clarifying, standardizing, cooling, homogenizing, bottling, and the various pasteurizing processes and sterilization; the modern theories of milk fermentations and the off-flavors of milk.

The second section of the book is more technical, and concerns the laboratory control of milk quality, including chemical, physical, and bacteriological tests.

Finally, there are two appendices. The first is a table showing the average chemical composition of Mexican feeds and the second summarizes the cost of milk production in the Mexico City area.

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